

THE EXPERIMENTAL MANIPULATION OF GONADCTROPHINS  
IN NON-PREGNANT SHEEP BY IMMUNIZATION AGAINST  
STEROID HORMONES

By

Nicholas David Martensz.



STATEMENT

The present studies were performed at the Medical Research Council Unit of Reproductive Biology, Edinburgh, under the supervision of Dr. R.J. Scaramuzzi and Professor R.V. Short.

The composition of this thesis and the interpretation of the experimental results were carried out solely by the author.

The work described in this thesis was performed by the author except where otherwise indicated in the text.

N.D. Martensz

March, 1977.



ACKNOWLEDGEMENTS

I would like first and foremost to express my gratitude to my supervisors, Dr. R.J. Scaramuzzi and Professor R.V. Short who introduced me to the many intrigues of reproductive biology and without whose encouragement and guidance the present studies would not have been completed.

Many thanks are also due to Drs. D.T. Baird and P.F.A. Van Look for their surgical expertise during the laparotomy experiments.

The assistance of Drs. R.J. Scaramuzzi and P.F.A. Van Look and Messrs. W. Davidson and I.J. Clarke in the collection of some of the blood samples during the long hours of the Scottish winter is very gratefully appreciated.

The radioimmunoassays for oestradiol, oestrone, androstenedione and testosterone were skillfully performed by Messrs. D.W. Davidson and I. Swanston.

Some of the illustrations were prepared by Mr. T. McFetters of the Department of Obstetrics and Gynaecology, Edinburgh University.

The routine management of the experimental animals was supervised by Mr. A. McGregor and the field staff of the A.B.R.O. Dryden Field Station, Roslin. In particular, the healthy condition of the experimental animals was largely due to the conscientious care of Mr. W. Davidson.

I would like to express my gratitude to the staff of the M.R.C. Unit of Reproductive Biology who provided a forum for the discussion of the experimental studies and who enabled the smooth running of the laboratory procedures.

The typing of this thesis was skillfully performed by Mrs.

S./

S. Edwards.

Last, but not least, I would like to thank my wife, Geraldine, for all her support and encouragement during the arduous task of research during the past three years.

The author received support through an M.R.C. Scholarship during the course of the present studies.

The involvement of a steroid in the feedback systems controlling the hypothalamic-adenohypophyseal axis may be inferred if a reduction in the biologically active fraction of the steroid through antibody binding results in an alteration in the secretion of gonadotrophins. The present studies examined the effects of active immunization against bovine serum albumin (BSA) conjugates of four steroids known to be secreted by the sheep ovary (oestradiol-17 $\beta$ , oestrone, androstenedione and testosterone) on the release of LH and FSH in the non-pregnant ewe.

Groups of five adult ewes were actively immunized against 17 $\beta$ -oestradiol-6-(O-carboxymethyl)oxime-BSA (E<sub>2</sub>-6-BSA), oestrone-6-(O-carboxymethyl)oxime-BSA (E<sub>1</sub>-6-BSA), testosterone-3-(O-carboxymethyl)oxime-BSA (T-3-BSA), androstenedione-11 $\alpha$ -hemisuccinyl-BSA (A-11-BSA) and BSA (controls). The concentrations of LH and FSH were measured in samples of jugular venous blood taken from all animals during anoestrus and during the breeding season. The ewes were laparotomized during the breeding season and samples of jugular venous and ovarian venous blood obtained for subsequent steroid analysis. The macroscopic appearance of the ovaries was noted and the ovaries were removed.

Ovine LH and FSH and oestradiol-17 $\beta$  (oestradiol), oestrone, androstenedione, testosterone and progesterone were all measured by specific radioimmunoassays. The binding of tritiated steroids by plasma was determined after the removal of the unbound steroid by dextran-charcoal absorption and after equilibrium dialysis.

The presence of elevated levels of LH together with the absence/

absence of behavioural oestrus and the inhibition of ovulation in four of the five oestradiol-immunized ewes served to confirm the widely accepted concept of a dual action of oestradiol on LH secretion in the ewe. Furthermore, multiple ovarian follicular development was evident in the four anovulatory oestradiol-immunized ewes and reflected the elevated circulating levels of LH.

A marked similarity between the oestradiol-immunized animals and the ewes immunized against T-3-BSA was apparent in terms of an elevated plasma level of LH, the absence of behavioural oestrus and the inhibition of ovulation accompanied by multiple follicular development. These findings coupled with the presence of significant oestradiol antibody titres and the failure of exogenous oestradiol to alter LH secretion during anoestrus in the testosterone-immunized animals led to the conclusion that immunization against T-3-BSA produced a non-specific reduction in the level of biologically active oestradiol.

Active immunization against E<sub>1</sub>-6-BSA produced plasma levels of LH greater than those found in ovariectomized-hysterectomized animals. A further anomalous finding in the present study was the absence of follicular stimulation in the oestrone-immunized ewes in response to the elevated circulating levels of gonadotrophins. The effects of active immunization against E<sub>1</sub>-6-BSA in the ewe could not be satisfactorily explained at the present time.

Active immunization against A-11-BSA produced an increased frequency of spontaneous discharges of LH during anoestrus and an elevation in the plasma level of LH during the luteal phase of the oestrous cycle. Furthermore, the positive feedback action of/

of oestradiol on LH secretion during anoestrus was delayed or absent in those ewes with high androstenedione antibody titres. Since the plasma binding of oestradiol was unaltered and oestrous cycles of normal length occurred in the androstenedione-immunized ewes, it is postulated that androstenedione, or its extra-ovarian metabolites, could modulate the feedback actions of oestradiol on LH secretion in the ewe.

Exogenous oestradiol exerts a dual action on the release of FSH in anoestrus and ovariectomized-hysterectomized ewes in a manner analagous to the negative and positive feedback actions of this steroid on LH secretion. A significant elevation in the plasma concentration of FSH was observed in the two oestradiol-immunized ewes with the highest titres of oestradiol antibodies. It is suggested that in these animals, the level of biologically active oestradiol was sufficiently reduced to lift the inhibitory control of FSH secretion. The presence of normal plasma FSH levels in the three remaining oestradiol-immunized animals suggests that incomplete neutralization of circulating oestradiol had left sufficient biologically active oestradiol available to exert an inhibitory action on FSH secretion.

In contrast, a reduction in the levels of biologically active androstenedione and testosterone in the ewes immunized against A-11-BSA and T-3-BSA failed to produce an elevation in the plasma level of FSH. In fact, a significant reduction in the plasma concentration of FSH was present in the androstenedione-immunized animals. It is tentatively suggested that androgens may enhance the release of FSH perhaps by a direct action of the anterior pituitary.

In the light of recent evidence suggesting that FSH may enhance/

enhance the aromatization of androgens to oestrogens by the ovary, it is postulated that the increased secretion of androgens by the ovary in response to LH may enhance the release of FSH. The elevated level of FSH would in turn lead to increased secretion of oestradiol thereby resulting in the suppression of LH and FSH secretion.

The problems associated with the evaluation of the antibody specificity and the degree to which the biological actions of steroids are neutralized in the actively immunized animal emphasize the need for caution in the extrapolation of the findings described in the present studies to physiological events in the intact animal. The proposed action of androgens on the release of FSH await further examination.

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CHAPTER 1

THE NEUTRALIZATION OF REPRODUCTIVE HORMONES

BY ANTIBODIES

Classical endocrinology has relied heavily on the degradative effects of endocrine gland removal (e.g. hypophysectomy or gonadectomy) and the restorative actions of hormone replacement therapy to delineate the physiological roles of hormones.

A third alternative, the neutralization of hormones by antibodies, in theory, offers a situation whereby individual hormones may be eliminated without alteration to the other components of the endocrine system. This technique would thus be superior to the classical methods, since hormone inter-actions, which may be important in modifying the feedback control systems in the intact animal, would remain unimpaired apart from the inhibited hormone. The present review describes the immunological studies examining the roles of reproductive hormones with particular reference to the neutralization of steroid hormones.

The concept of hormone inhibition by antibodies was recognized as long ago as the 1930's (see reviews by Collip, 1935; Collip et al. 1940; Thompson, 1941; Zondek & Sulman, 1942) when prolonged treatment with gonadotrophin preparations were found to produce a state of refractoriness towards further gonadotrophic stimulation. These early studies, however, were hampered by the relatively impure pituitary gonadotrophin preparations used and the subsequent production of non-specific antibodies. It was not until the 1960's when the availability of purified gonadotrophins and the advent of radioimmunoassays stimulated research into the physiological effects of hormone antibodies.

#### 1.1. The effects of antibodies to gonadotrophins in male animals.

Passive immunization of male rats with an antiserum to ovine luteinizing hormone (LH) resulted in regression of the accessory glands and testes accompanied by spermatogenic arrest (Hayashida, 1963).

Similar effects were found in male rats actively immunized with

ovine LH (Wakabayashi & Tamaoki, 1966). Moreover, in vitro biosynthetic studies demonstrated a reduction in the activity of the enzymes involved in androgen synthesis (Wakabayashi & Tamaoki, 1966). Furthermore, there was some evidence to suggest that LH synthesis was increased since a proliferation of basophilic cells was observed in the pituitaries from the LH-immunized rats together with an increased incorporation of  $^{14}\text{C}$ -leucine into the LH fraction (Wakabayashi & Tamaoki, 1966). This last finding may have been due to the reduction in androgen secretion, since high levels of gonadotrophins are present in the blood of castrate animals.

Active immunization of male rabbits with ovine LH (Wakabayashi & Tamaoki, 1966; Quadri, Harbers & Spies, 1966) or bovine LH (Talaat & Laurence, 1971) also produced a reduction in libido and atrophy of the accessory glands and testes accompanied by aspermiogenesis. Testosterone was effective in overcoming the loss of libido but failed to restore testicular function (Quadri et al. 1966).

In the light of these studies, the inhibition of LH by antibodies resulted in the atrophy of the testes and accessory glands together with a loss of libido and suggested that LH acted by promoting androgen secretion. In this respect, these studies corroborated the classical study of Greep, Fevold and Hisaw (1936) on the restorative effects of LH in the hypophysectomized male rat. However, the neutralization of LH also interrupted spermatogenesis and altered the tubular elements of the testes (Wakabayashi & Tamaoki, 1966). It is tempting to explain this apparent action of LH on the follicle-stimulating hormone (FSH) dependent processes (Greep et al. 1936) by the presence of cross-reacting antibodies causing partial neutralization of FSH.

An attempt to resolve this problem by active immunization of prepubertal male rabbits with ovine FSH (Monastirsky, Laurence & Torvar, 1971)/

1971) gave inconclusive results. The testes weight was unaltered and histological examination revealed complete spermatogenesis and normal interstitial tissue. Although antibodies to ovine FSH were demonstrated, no evidence was given to establish the neutralization of endogenous rabbit FSH.

On the other hand, Turner and Johnson (1971) found reduced fertility without loss of libido in male rats passively immunized with an antiserum to ovine FSH. Histological examination of the male reproductive tract revealed normal testicular tissue with normal spermatogenesis. However, some alteration in the epithelial lining of the epididymis occurred. In this study, the titre of FSH antibodies was not determined and it is difficult to evaluate the completeness of FSH inhibition.

#### 1.2. The effects of gonadotrophin deprivation in female animals.

Immunological studies with FSH and LH antibodies have attempted to delineate the roles of gonadotrophins in ovulation, follicular maturation and pregnancy in female animals.

Active immunization with either LH (Laurence & Ichikawa, 1968) or FSH (Talaat & Laurence, 1969) produced marked, but highly variable alterations in the reproductive processes of the female rat.

Immunization against LH increased the length of the oestrous cycle, generally by prolonging the dioestrus phase of the cycle (Laurence & Ichikawa, 1968). Histological examination of the ovaries revealed a reduction in the size and number of corpora lutea and the presence of large atretic follicles (Laurence & Ichikawa, 1968) suggesting that to some extent ovulation had been inhibited without alteration in follicle development. Moreover, their fertility was reduced by an increased incidence of prolonged anoestrus, sterile mating/

mating or a reduction in implantation (Laurence & Ichikawa, 1968).

Abnormal oestrous cycles and a reduced reproductive capacity were also observed in rats actively immunized with ovine FSH (Talaat & Laurence, 1969). The ovaries, however, showed a marked inhibition of follicular maturation with the presence of multiple follicular cysts.

The complex interrelationships in the female reproductive processes made the interpretation of these long-term inhibition studies difficult. Passive immunization, on the other hand, enabled the short-term inhibition of hormones, thereby allowing some control over the period of hormone inhibition.

The ovulatory role of LH in the cycling female rat was confirmed by the inhibition of ovulation in rats treated with anti-LH sera on the day of proestrus (Kelly, Robertson & Stansfield, 1963; Schwartz & Gold, 1967). Moreover, a reduction in the pituitary LH content on the morning of oestrus suggested that the antibodies had neutralized the preovulatory LH surge after its release from the pituitary.

The blockade of ovulation by LH antibodies has also been demonstrated in the mouse (Ely, Tuercke & Chen, 1966), rabbit (Quadri, Harbers & Spies, 1966; Pineda, Faulkner, Hopwood & Luecker, 1968), hamster (Jagannadha Rao, Moudgal, Madhwa Raj, Lipner & Greep, 1974), pig (Spies, Styler & Quadri, 1967) and cow (Snook, Brunner, Saatman & Hansel, 1969).

Rats treated with LH antisera on the day before proestrus not only failed to ovulate (Bourdel & Li, 1963; Ely & Schwartz, 1971) but also failed to display the oestrogen-dependent uterine distension and vaginal cornification (Ely & Schwartz, 1971). Furthermore, no reduction in the pituitary LH content was found on the morning of oestrus (Ely & Schwartz, 1971) suggesting that the inhibition of LH on/



on the day before proestrus had prevented the release of the preovulatory LH surge by lowering the level of circulating biologically active oestrogens. This study further promoted the concept of an oestrogen - trigger in the ovulatory process in the rat.

Although the absolute requirement for LH in the ovulatory process is well established, the role of FSH in this process remains undefined.

Goldman and Mahesh (1969) found that an anti-LH serum, absorbed with FSH to remove FSH activity, failed to inhibit ovulation in the hamster. This finding suggested that FSH rather than LH was an absolute requirement for ovulation in this species.

In contrast, however, Jagannadha Rao et al. (1974) found that an anti-FSH serum was ineffective in preventing LH-induced ovulation in the immature hamster.

Furthermore, LH antisera were effective in preventing FSH-induced ovulation in the immature hamster (Jagannadha Rao et al. 1974), rat (Lipner, Hirsch, Moudgal, MacDonald, Shao-Yao Ying & Greep, 1974) and phenobarbital-blocked rat (Cobbs, Schwartz & Ely, 1972) indicating that the LH antibodies had neutralized the LH contamination in the FSH preparation. Moreover, an anti-ovine FSH serum failed to inhibit ovulation or vaginal cornification when given to rats at proestrus although some alteration in follicular development was apparent during the next cycle (Schwartz, Krone, Talley & Ely, 1973).

These studies suggest that ovulation may occur without FSH and the primary role of the preovulatory FSH surge may be the initiation of follicular development in the next cycle. This concept was further illustrated in a recent study by Welschen and Dullaart (1976). Treatment of proestrus rats with an anti-FSH serum failed to inhibit ovulation but reduced follicular development in the next cycle whereas an/



an LH antiserum inhibited ovulation but had no effect on follicle development in the following cycle (Welschen & Dullaart, 1976).

The dependence of implantation and the maintenance of early pregnancy on the pituitary has been confirmed by the finding that passive immunization with LH antisera during early pregnancy in rats (Loewit, Badaway & Laurence, 1969; Madhwa Raj & Moudgal, 1970), mice (Munshi, Pundare & Rao, 1972), rabbits (Spies & Quadri, 1967) and hamsters (Jagannadha Rao, Madhwa Raj & Moudgal, 1970, 1972) resulted in abnormal gestation with an increased incidence of foetal resorption. Moreover, a single injection of an LH antiserum on day 4 of gestation in the rat delayed implantation (Madhwa Raj, Sairam & Moudgal, 1968), suggesting that LH was an absolute requirement for implantation in the rat.

A reduction in ovarian progesterone secretion in rats treated with an LH antiserum during early pregnancy (Moudgal, Behrman & Greep, 1972) suggested that the gestational abnormalities were due to a reduction in the gonadotrophic support for the corpus luteum and a subsequent reduction in steroid secretion. This concept was further corroborated by the finding that progesterone largely overcame the effects of LH antisera on early gestation although this reversal was more complete when both progesterone and oestrogen were given (Spies & Quadri, 1967; Jagannadha Rao et al. 1972; Munshi et al. 1972).

These immuniological studies firmly established the requirement of LH during early pregnancy in laboratory animals. The requirement for FSH and prolactin, however, is less clear, since neither FSH nor prolactin were effective in overcoming the effects of LH antisera during early pregnancy (Madhwa Raj & Moudgal, 1970; Jagannadha Rao et al. 1972).

1.3. The immunological neutralization of the hypothalamic gonadotrophin-releasing hormone(s).

The isolation of the decapeptide, luteinizing hormone-releasing hormone (LHRH) with both LH and FSH releasing activity from porcine (Schally, Arimura, Baba, Nair, Matsuo, Redding, Debeljuk & White, 1971) and ovine (Amoss, Burgus, Blackwell, Vale, Fellows and Guillemin, 1971) hypothalamic tissue together with its synthesis (Geiger, Konig, Wissman, Geisen & Enzmann, 1971; Matsuo, Arimura, Nair & Schally, 1971) has added a new dimension to reproductive endocrinology.

The existence of a separate follicle-stimulating hormone releasing hormone (FSHRH) has been the subject of some contention. Purification of porcine hypothalamic tissue by gel filtration has yielded fractions which contained considerably higher FSH releasing activity than LHRH itself (Currie, Johansson & Folkers, 1973; Johansson, Currie & Folkers, 1973; Bowers, 1973) and suggested the existence of a separate FSHRH. On the other hand, Schally et al. (1971) were unable to separate the FSH releasing activity from LH releasing activity by partition chromatography and concluded that the LHRH decapeptide was responsible for the physiological release of both gonadotrophins.

The immunogenic properties of conjugates of LHRH have led to the production of antibodies to LHRH (Arimura, Sato, Kumasaka, Worobec, Debeljuk, Dunn & Schally, 1973b; Jeffcoate, Holland, Fraser & Gunn, 1974b; Jeffcoate, Fraser, Holland & Gunn, 1974a; Arimura, Sato, Coy, Worobec, Schally, Yanaihara, Hashimoto, Yanaihara & Sukura, 1975).

Passive immunization with LHRH antisera prevented ovulation in rats when given at proestrus (Arimura, Debeljuk & Schally, 1973a; Fraser & Gunn, 1973; Koch, Chobsieng, Zor, Fridkin & Lindner, 1973). Moreover/

Moreover, the plasma concentrations of LH and FSH in blood taken on the afternoon of proestrus were very low indicating that the preovulatory gonadotrophin surge had been prevented. These studies provide evidence for the involvement of LHRH in the release of the preovulatory gonadotrophin surge in the intact rat.

Active (Fraser, Gunn, Jeffcoate & Holland, 1974; Arimura, Shino, De la Cruz, Rennels & Schally, 1976) or passive (Arimura et al. 1976) immunization of intact (Fraser et al. 1974) or castrate (Arimura et al. 1976) male rats or rabbits against LHRH resulted in a marked reduction in the plasma and pituitary concentrations of both LH and FSH together with a marked atrophy of the testes and accessory glands accompanied by spermatogenic arrest.

A reduction in the plasma and pituitary concentrations of both LH and FSH was also found in ovariectomized rats actively immunized with LHRH (Fraser, Jeffcoate, Gunn & Holland, 1975).

The reduction in both LH and FSH in animals actively immunized against LHRH provides additional evidence for the concept of a single gonadotrophin-releasing hormone. However, the possibility that a separate FSHRH, immunologically identical with LHRH, does exist cannot entirely be ruled out.

#### 1.4. Immunization against human chorionic gonadotrophin (HCG):

##### A new form of anti-fertility agent?

The search for a new anti-fertility agent has recently been directed towards the prevention of implantation and the maintenance of early pregnancy by immunization against HCG, the gonadotrophin of pregnancy.

Early studies, however, showed that immunization against intact HCG resulted in the disruption of the menstrual cycle in women (Stevens & Crystle, 1973; Stevens, 1974, 1975 a,b) due to a cross reaction with/

with human LH. This finding is not surprising since it has been demonstrated that the  $\alpha$ -subunit of HCG is identical with the  $\alpha$ -subunits of LH, FSH and thyroid-stimulating hormone (TSH) (Canfield, Morgan, Kammerman, Bell & Agosto, 1971; Pierce, Liao, Howard, Shome & Cornell, 1971).

The  $\beta$ -subunit of HCG ( $\beta$ -HCG), however, is hormone-specific and offers a situation whereby antibodies raised against this polypeptide would have a low cross-reaction with human LH.

Stevens (1974) passively immunized a pregnant baboon with an ovine anti- $\beta$ -HCG serum on day 20 of gestation and produced an abortion about 36 hours later. Moreover, baboons actively immunized against  $\beta$ -HCG failed to have normal pregnancies after a total of 30 matings even though neither the menstrual cycle nor ovulation were affected (Stevens, 1974; 1975 a,b). However, antisera raised against  $\beta$ -HCG in the rabbit or baboon still showed a significant cross-reaction with human LH (Stevens, 1975b) and were considered unsuitable for use as an anti-fertility agent in women. Stevens (1975 a,b) is now studying the cross reactivity of antisera raised against fragments of the  $\beta$ -HCG polypeptide, particularly synthetic polypeptides containing the 30-32 C-terminal amino acids which have no analogous counterpart in the  $\beta$ -subunit of human LH, in an attempt to produce antibodies with minimal cross-reactivity with human LH.

Independently, Hearn has successfully terminated pregnancy by the induction of early abortions in marmoset monkeys actively or passively immunized against  $\beta$ -HCG without alteration in the normal menstrual cycle (Hearn, Short & Lunn, 1975; Hearn, 1976).

Talwar et al, have successfully raised antibodies to  $\beta$ -HCG in women and rhesus monkeys actively immunized with  $\beta$ -HCG conjugated/

conjugated to tetanus toxoid. No harmful side-effects were observed and endometrial biopsies and luteal phase progesterone levels suggested that there was no impairment of ovulation or luteal function (cited by Hearn, 1976).

Although still in the early stages of development, with the problems of irreversibility and toxic effects of the immunization procedure to be completely eliminated, immunization against  $\beta$ -HCG, or fragments of this peptide, might well be a practical form of human population control in the future.

#### 1.5 The neutralization of steroid hormones by antibodies.

The steroid hormones are compounds of low molecular weight (200-400) and as such are not immunogenic. Early attempts to induce antibody formation in rats by prolonged oestrogen treatment were unsuccessful (D'Armour, Dumont & Gustavson, 1934). Perhaps the first report of the neutralization of the physiological actions of steroid hormones appeared in 1937 when Toby and Lewis (1937) found that dogs receiving repeated injections of adrenocortical extracts became refractory towards further treatment. Moreover, the inhibitory factor was transmitted in the serum of refractory animals (Toby & Lewis, 1937). However, the failure of crystalline desoxycorticosterone or corticosterone to induce an inhibitory state (Hartman, Lewis & Gabriel, 1940) suggested that the antibodies were not directed against the steroid hormones themselves but rather against a protein present in the adrenocortical extracts.

Landsteiner (1936) established the "hapten" concept whereby immunization with a small compound, with no inherent immunogenic properties, conjugated to a larger foreign protein, could induce the formation of antibodies directed not only against the carrier but/

but also to the attached organic moiety.

In the late 1950's, Lieberman and his associates utilized the hapten principle and reported the preparation of several steroid-protein conjugates (Erlanger, Borek, Beiser & Lieberman, 1957, 1959) and demonstrated the presence of antibodies to both the carrier protein and the steroid hapten in the sera of immunized rabbits (Lieberman, Erlanger, Beiser & Agate, 1959). The conjugates also possessed hormonal properties (Lieberman et al. 1959).

The specificity of the antibodies, assessed by a hapten inhibition test, suggested that the anti-steroid antibodies were directed against the parts of the steroid molecule distal to the site of conjugation (Lieberman et al. 1959).

Passive immunization of castrate rats with antisera to testosterone-17-hemisuccinyl-BSA (T-17-BSA) inhibited the increase in the weights of the seminal vesicles and ventral prostate in response to implants of the conjugate suggesting that the antibodies could neutralize the physiological actions of exogenous steroids (Lieberman et al. 1959). In a more extensive study, Neri, Tolksdorf, Beiser, Erlanger, Agate & Lieberman (1964) confirmed the inhibition of the action of exogenous testosterone, oestrone, cortisol and aldosterone by antisera to their respective conjugates.

Independently, Goodfriend and Sehon prepared an oestrone conjugate (Goodfriend & Sehon, 1958) and neutralized the uterotrophic action of oestrone in immature rats with antisera raised against this conjugate (Goodfriend & Sehon, 1961).

Early attempts to neutralize the action of endogenous hormones by active immunization were unsuccessful. The Schering group/



group failed to demonstrate any alteration in the development of the combs of young cockerels actively immunized with either testosterone-17-BSA or testosterone-3-(O-carboxymethyl)oxime-BSA (T-3-BSA) within 4 weeks of the start of immunization (cited by Lieberman et al. 1959). The inability of the anti-testosterone antibodies to neutralize the endogenous steroid may be attributed to the relatively short period over which the study was performed. This was further corroborated by the finding that the hormonal effect of T-17-BSA on the seminal vesicles of the castrate rat was counteracted only after 24 days of treatment with the conjugate (Lieberman et al. 1959).

Studies with steroid-protein conjugates lapsed after these early studies until the late 1960's when the advent of steroid radioimmunoassays brought forth a plethora of renewed interest. Although it is beyond the scope of the present study to review in depth the literature pertaining to steroid radioimmunoassays, it is perhaps relevant to outline some of the studies concerning the specificity of steroid antibodies.

The steroid-protein conjugates prepared by Lieberman and his colleagues and Goodfriend and Schon utilized the existing functional groups on the native steroid molecule to form derivatives for subsequent conjugation. The preparation of steroid derivatives at sites distal to functional groups enables the formation of steroid-protein conjugates which left the functional groups free to act as antigenic determinants (Dean, Exley & Johnson, 1971; Lindner, Perel, Friedlander & Zeitlin, 1972; Walker, Clark & Wotisz, 1973; Rosenfeld, Rosenberg, Kream & Hellman, 1973; Condom & Emiliozzi, 1974; Pang & Johnson, 1974; Kellie, Lichman & Samarajeewa, 1975; Kohen, Bauminger & Lindner, 1975).

Conjugation of oestradiol-17 $\beta$  at the C-6 position

(E<sub>2</sub>-6-BSA) left the functional groups free. Antibodies induced to this conjugate showed a greatly reduced cross-reaction with closely related oestrogens (oestradiol-17 $\alpha$ , oestrone and oestriol, less than 4%) (Exley, Johnson & Dean, 1971; Lindner et al. 1972; Wright, Collins & Preedy, 1973; Den Hollander, Van Weeman & Woods, 1974; Niswender, Nett, Meyer & Hagerman, 1975) and showed a greater specificity than antibodies raised against oestradiol-protein conjugates coupled at the C-17 or C-11 positions (Lindner et al. 1972; Wright et al. 1973; Den Hollander et al. 1974).

Conjugation of progesterone at the C-11 position induced the formation of antibodies with greater recognition of the A and D rings than antibodies to progesterone-6-BSA (Lindner et al. 1972; Niswender, 1973).

Conjugation of testosterone at the C-3 or C-11 positions induced antibodies with similar specificities (Hillier, Brownsey & Cameron, 1973a; Bosch, Den Hollander & Woods, 1974).

It is apparent from these studies that, in general, antibodies are directed at parts of the molecule distal to the site of coupling. It should however be noted at this point that the above studies related to antibody specificity in the radio-immunoassay context. The commonly used method for determining the specificity (Abraham, Odell, Edwards & Purdy, 1970) in such cases actively selects a high antiserum dilution thereby negating the effects of high affinity, low titre antibodies. These studies therefore cannot be related to the plasma steroid binding in vivo and are of limited value in assessing the specificity of antibodies in physiological studies.

The/



The neutralization of steroids by passive or active immunization exerts widespread effects on the reproductive processes in experimental animals.

Ferin, Zimmering, Lieberman and Vande Wiele (1968) firmly established the concept of neutralization of circulating oestrogens in passively immunized rodents. The uterotrophic action of exogenous oestrogen in immature mice and adult ovariectomized rats was inhibited by an antiserum to oestradiol-17-hemisuccinyl-BSA ( $E_2$ -17-BSA) (Ferin et al. 1968). Neutralization of endogenous oestrogens by the antiserum was confirmed by the inhibition of the uterine response to HCG in immature rats (Ferin et al. 1968). Furthermore, the ovarian weight increase in response to HCG was not affected by the antiserum suggesting that the antibodies did not inhibit the synthesis of oestrogens. The failure of the antiserum to inhibit the oestrogenic action of the non-steroidal compound, diethylstilboestrol (DES) (Ferin et al. 1968) suggested that the antibodies acted by neutralizing circulating oestrogens rather than by combining irreversibly with oestrogen receptors on the target organs.

Using two models, the PMS-treated immature rat and the adult cycling rat, Ferin examined the role of oestrogens in the ovulatory process.

Immature female rats treated with a single injection of PMS released the preovulatory gonadotrophin surge some 50 hours later and then ovulated after a further 12 hours (Ferin, Zimmering & Vande Wiele, 1969a). Treatment with an oestradiol antiserum within 28 hours of the PMS injection inhibited ovulation (Ferin et al. 1969a). Furthermore, the oestrogen-dependent uterine distension and vaginal cornification was prevented by the antiserum suggesting

a deficiency of circulating oestrogens (Ferin et al. 1969a).

In cycling rats, a rise in the plasma oestrogen concentration occurs on the day before proestrus (Yoshinaga, Hawkins & Stocker, 1969). Rats treated with a single injection of the anti-E<sub>2</sub>-17-BSA serum at 1000h on the day before proestrus prevented uterine ballooning on the morning of proestrus and the appearance of cornified epithelial cells in the vaginal smear (Ferin, Tempone, Zimmering & Vande Wiele, 1969b). These findings supported the concept of an oestrogenic trigger in the ovulatory process.

In both the PMS-treated immature rat and the cycling rat, ovulation in response to DES or HCG was not affected by the presence of oestrogen antibodies (Ferin et al. 1969a,b), suggesting that the ovarian response to gonadotrophins was impaired only by the neutralization of circulating biologically active oestrogens.

The failure of treatment with an antiserum to progesterone on the second day of dioestrus to prevent ovulation in the cycling rat (Ferin et al. 1969b) cast some doubt on the absolute requirement of this steroid in the ovulatory process.

These studies confirmed the effects of anti-LH sera given on the day before proestrus in inhibiting the rise in oestrogens and subsequently, the gonadotrophin surge and ovulation.

Passive immunization with antisera to oestrogens or progesterone has also aided in the delineation of the role of these steroids in pregnancy in the rat. Treatment with a single injection of either antisera on day 3 or 4 of gestation delayed implantation by 24 to 96 hours as determined by the presence of free ova in uterine lavages taken on days 6 or 7 (Raziano, Ferin & Vande Wiele, 1972). Subsequent laparotomy on days 9 and 17 revealed/

revealed a reduction in the size of implantation sites after treatment with either antisera, and reabsorption of fetuses in animals receiving progesterone antiserum on days 3 or 4, or the oestradiol antiserum on day 4 (Raziano et al. 1972). These findings suggested that not only are progesterone and oestrogen important in the implantation process, but that hormonal disturbances during early pregnancy may have long-term effects on the course of pregnancy. Whereas treatment with either antisera on days 7, 13 or 15 had no effect on normal pregnancy, administration of the progesterone antiserum, and to a lesser extent, the oestradiol antiserum, on day 11 resulted in reabsorption of the fetuses (Raziano et al. 1972).

In a quantitative study, Dray, Csapo and Erdos (1975) found that an anti-progesterone serum given to rats on day 10 of gestation caused abortion within about 48 hours whereas the same treatment given on day 6 had no effect. Measurement of unbound progesterone showed a reduction from 45ng/ml to 15ng/ml in the 6 hour period following treatment on day 10, but a smaller reduction from 65ng/ml to 45ng/ml occurred on day 6. (Dray et al. 1975.)

These findings suggest that the effectiveness of steroid neutralization may depend on the level of endogenous steroid at the time of antiserum administration. It is also possible that the failure of the progesterone and oestrogen antisera to affect gestation when given on day 7 (Raziano et al. 1972) may have been due to a high level of endogenous progesterone and subsequently, the incomplete neutralization of endogenous steroid.

Treatment of progesterone-primed ovariectomized ewes with an antiserum to oestradiol-17 $\beta$  prevented the expression of oestrous behaviour/

behaviour in response to oestradiol benzoate (Scaramuzzi, 1975a). On the other hand, administration of the anti-oestradiol antiserum to intact, cycling ewes on days 12 to 15 of the cycle failed to alter normal luteal regression, the length of the cycle or the expression of oestrous behaviour (Scaramuzzi, 1975b). Subsequent histological examination of the ovaries from the immunized ewes showed signs of thecal stimulation. Scaramuzzi (1975b) suggested that compensatory oestradiol secretion had led to the saturation of the limited amount of antibody and consequently a pool of biologically active oestradiol was available to exert its normal physiological functions.

In a more recent study, a high dose of an oestradiol antiserum (25ml twice daily) during the latter days of the oestrous cycle in the ewe prevented ovulation and the expression of oestrous behaviour (Fairclough, Smith & Peterson, 1976a; Fairclough, Smith, Peterson & McGowan, 1976b).

It is apparent that passive immunization studies, although enabling some control over the duration of antibody action, must utilize a titre of antibodies sufficient to neutralize the endogenous steroid. This necessitates not only a full characterization of the antiserum but also some knowledge of the steroid secretion in the animal to be studied.

Active immunization, in theory, offers a situation whereby high affinity antibodies may be present for long periods, thereby realizing the neutralization of endogenous steroids throughout the experimental period.

The first studies to utilize active immunization against steroids demonstrated the absence of the positive LH response to oestradiol benzoate in progesterone-primed ovariectomized ewes actively/

actively immunized with  $E_2$ -17-BSA (Caldwell, Scaramuzzi, Tillson & Thorneycroft, 1970; Scaramuzzi, Caldwell, Tillson & Thorneycroft, 1970a).

Active immunization against oestrogens disrupted the oestrous cycle in hamsters (Caldwell et al. 1970) and rats (Hillier, Groom, Boyns & Cameron, 1975a,b) and the menstrual cycle in rhesus monkeys (Cowchock, Ferin, Dyrenfurth, Carmel, Zimmerman, Brinson & Vande Wiele, 1973; Sundaram, Tsong, Hood & Brinson, 1973).

In female rats actively immunized with  $E_2$ -6-BSA, antibodies were present in the serum between 6 and 16 weeks after the start of the immunization procedure (Hillier et al. 1975a,b). During this period, the total oestradiol concentration (both free and bound) rose dramatically. Although the percentage of oestradiol bound by the plasma, determined by equilibrium dialysis, increased, extrapolation of the data to the total concentration yielded a net increase in the level of "free" oestradiol (Hillier et al. 1975a,b). The large error associated with the measurement of very high binding, inherent in the equilibrium dialysis method, however, may not have resulted in an accurate reflection of the true situation. The elevation of plasma LH, but not FSH or prolactin (Hillier et al. 1975a,b) further suggested that the level of free oestradiol was reduced, thereby lowering the negative feedback action of this steroid. The ovaries from the oestradiol-immunized female rats revealed few corpora lutea and the presence of multiple follicular cysts (Hillier et al. 1975a,b). This finding suggested an anovulatory condition and was further supported by the presence of cornified cells in vaginal smears taken on 8 consecutive days before death (Hillier et al. 1975a,b).

The/

The differential effects of oestradiol immunization in female rats suggests that this steroid may have different feedback actions on the secretion of LH and FSH. There is some evidence in humans to suggest that FSH is more sensitive to the negative feedback action of oestrogens since FSH was suppressed to a greater degree than LH in women (Tsai & Yen, 1971) and men (Kulin & Reiter, 1972) after treatment with ethinyl-oestradiol. However, in rats, LH is suppressed more rapidly than FSH after treatment of castrate animals with oestradiol (Mahesh, Muldoon, Eldridge & Korach, 1975). However, the longer half life of FSH in the plasma of hypophysectomized rats (Gay, Midgley & Niswender, 1970) could explain this effect.

Plasma from female rats actively immunized with T-3-BSA showed an elevation in the total plasma testosterone concentration accompanied by a reduction in the level of "free" testosterone (Hillier, Groom, Boyns & Cameron, 1974). In contrast to the oestradiol-immunized female rats, these animals showed an elevation in plasma FSH without alteration in the levels of LH or prolactin (Hillier et al. 1974). Vaginal cyclicity was absent and the vaginal smear characterized by the presence of cornified cells. The ovaries were enlarged and contained multiple follicular cysts characterized by the almost total absence of granulosa cells (Hillier et al. 1975a,b).

The differential effects on gonadotrophin secretion in the testosterone-immunized female rat also suggests a differential action of testosterone on gonadotrophin secretion in the female. It is, however, rather surprising that vaginal cyclicity was disrupted assuming there was no cross-reaction with oestrogens. The specificity of antibodies in vivo will be discussed later in this/



this review.

A similar anovulatory condition to that found in oestradiol-immunized rats was also present in rhesus monkeys actively immunized with either an oestrone conjugate (Sundaram et al. 1973) or an oestradiol conjugate (oestradiol-17-BSA) (Cowchock et al. 1973). The anovulatory condition was determined by the absence of luteal levels of progestins and an increased interval between menstruation. The degree of anovulation was positively correlated with the titre of oestrogen antibodies (Sundaram et al. 1973; Schwartz, Dyrenfurth, Khalaf, Vande Wiele & Ferin, 1975).

Further studies on the oestradiol-immunized rhesus monkeys revealed a significant elevation in the plasma concentration of LH, although the levels failed to reach those found in ovariectomized monkeys (Ferin, Dyrenfurth, Cowchock, Warren & Vande Wiele, 1974). Histological examination of the ovaries revealed the presence of multiple follicular cysts and an absence of corpora lutea (Ferin et al. 1974).

In contrast to oestradiol immunization, active immunization of female rhesus monkeys with progesterone-20-BSA failed to alter the interval between menstruation, and ovulation, determined by the occurrence of luteal levels of progestins, occurred in about 50% of these cycles (Schwartz et al. 1975). Hormonal levels measured in daily blood samples throughout two of these ovulatory cycles revealed the normal late follicular rise in plasma oestrogens preceding the preovulatory LH surge and the subsequent rise in progestins with the formation of the corpus luteum (Schwartz et al. 1975). These findings together with the failure of an anti-progesterone serum to prevent ovulation in rats (Ferin et al. 1969a) suggested that progesterone was not an absolute requirement for/

for the ovulatory process in these species.

In male rabbits and rats actively immunized with T-3-BSA, the total plasma testosterone concentration was markedly elevated and the percentage of steroid bound increased to almost 100% (Hillier, Cole, Groom, Boyns & Cameron, 1973b; Nieschlag, Usadel, Schwedes, Kley, Schoffling & Kruskemper, 1973; Nieschlag, & Kley, 1974; Nieschlag, Tekook, Usadel, Kley & Kruskemper, 1975a; Nieschlag, Usadel, Wickings, Kley & Wuttke, 1975b). The apparent reduction in the circulating level of biologically active testosterone resulted in an elevation in the plasma concentrations of both LH and FSH (Hillier et al. 1973b; Nieschlag et al. 1975b).

Hypertrophy of the testes as a result of the increased gonadotrophin levels was apparent in testosterone-immunized animals (Nieschlag et al. 1973; Hillier et al. 1975a,b). Histological examination of the testes revealed an increase in the interstitial tissue characterized by the proliferation of Leydig cells (Nieschlag et al. 1973; Hillier et al. 1975 a,b). The testicular testosterone concentration in testosterone-immunized rabbits and the testicular response to HCG in vitro increased by the same order of magnitude as the mass of interstitial tissue (Nieschlag et al. 1975a). Thus the endocrine capacity of the testes was augmented parallel to the increase in Leydig cell tissue.

The presence of complete spermatogenesis in the testes of testosterone-immunized rabbits (Nieschlag et al. 1973) suggested that testosterone acted on the tubules before entering the blood where it was bound by antibodies.

The reduction in biologically active testosterone was confirmed by the loss of libido in rabbits actively immunized with T-3-BSA (Nieschlag & Kley, 1974) and atrophy of the accessory glands /



glands (Nieschlag et al. 1973). The increased levels of both LH and FSH after the elimination of biologically active testosterone confirmed the well-documented role of testosterone in testicular feedback.

In contrast to the results cited above, active immunization of male rats with  $E_2$ -6-BSA failed to alter plasma gonadotrophin levels although the total concentration and binding of oestradiol were increased (Hillier et al. 1975a,b). The lack of effect on gonadotrophin levels was attributed to the presence of a small pool of biologically active oestradiol (Hillier et al. 1975 a,b). It is however, hard to accept the 1,000 fold increase in the total plasma oestradiol concentration (Hillier et al 1975a,b) unless some compensatory secretion of this steroid had occurred presumably as a result of increased gonadotrophic stimulation of the testes. However, neither the weight nor the histology of the testes from the oestradiol-immunized male rats differed from the control tissues (Hillier et al. 1975a,b).

Independently, Nieschlag examined the effects of active immunization of male rabbits with  $E_2$ -6-BSA (Nieschlag, Usadel, Kley, Schwedes, Schoffling & Kruskemper, 1974; Nieschlag et al. 1975b). All animals showed an elevated total plasma oestradiol concentration accompanied by increased steroid binding. The level of LH was significantly higher than the control animals and no alteration in the level of FSH occurred. The percentage of Leydig cells in the testes of the oestradiol-immunized male animals was increased but no alteration in the tubules was apparent. Furthermore, the weight of the testes from the oestradiol-immunized rabbits was not increased thereby reflecting the unaltered plasma FSH levels.

It is difficult to evaluate the conflicting results in oestradiol/

oestradiol-immunized animals. It would appear from Nieschlag's work that oestradiol exerted a negative feedback action on LH secretion in the male.

Active immunization studies have revealed an elevation in the plasma concentration of the steroid against which the animal was immunized. The increased steroid levels in animals immunized against oestradiol or testosterone may be the result of two factors, increased gonadal steroid secretion in response to the elevated gonadotrophin levels and/or a reduction in the clearance of antibody-bound steroid from the circulation.

Sundaram et al. (1973) and Schwartz et al. (1975) demonstrated an increased retention of tritiated oestrogen in the blood of oestrogen-immunized monkeys.

In a more detailed study, Wickings, Becher and Nieschlag (1976) examined the metabolism of testosterone in male rabbits actively immunized with T-3-BSA. After a single injection of  $^3\text{H}$ -testosterone, the metabolic clearance rate was 1/10th that seen in control animals. Furthermore, the half-life of testosterone, determined by the decline in endogenous plasma testosterone levels after castration, was twice as long in the testosterone-immunized animals (Wickings et al. 1976).

Histological evidence of ovarian and testicular stimulation have already been described. Moreover, the production rate of testosterone in testosterone-immunized animals was increased (Nieschlag et al. 1975a; Wickings et al. 1976).

It is apparent therefore, that the elevated steroid levels result from a combination of decreased steroid clearance and an increased steroid production rate. This observation is further corroborated by the study of rhesus monkeys actively immunized against/

against progesterone (Schwartz et al. 1975). In these animals, the clearance of a pulse injection of  $^3\text{H}$ -progesterone was reduced but an elevation of progestin levels was not encountered. The occurrence of normal plasma LH concentrations during the menstrual cycle suggests that progesterone does not play an active role in the negative feedback control of LH secretion. Consequently, gonadotrophin-stimulated ovarian steroid hypersecretion would not have occurred thus removing one of the factors responsible for increased steroid levels.

The presence of large amounts of steroid casts some doubt on the completeness of steroid neutralization. The presence of cornified cells in the vaginal smears from oestradiol-immunized monkeys, together with the failure of LH levels to reach those seen in ovariectomized animals (Ferin et al. 1974), suggests the presence of some oestrogenic action in these animals. The equilibrium nature of the antibody-steroid inter-action, however, is such that complete, irreversible binding of steroids would be difficult to achieve. Moreover, the relative antibody and steroid receptor affinities may be such that the receptors could compete successfully for the circulating steroid. This hypothesis has received some support from the studies by Hillier and Cameron (1975a,b) on the effects of oestradiol immunization on hormone-dependent mammary carcinomas in the rat.

Carcinomas induced by 7,12-dimethylbenz(a)anthracene (DMBA) will regress after hypophysectomy or ovariectomy (Huggins, Briziarelli & Sutton, 1959). Furthermore, tumour growth can be restored in ovariectomized-adrenalectomized rats by treatment with oestradiol (Pearson, Murray, Mozaffarian & Pensky, 1972). It is conceivable, therefore, that the neutralization of circulating oestradiol/

oestradiol by antibodies could cause regression of the tumours in the intact animal. However, both active and passive immunization in female rats enhanced the mammary tumours induced by DMBA (Hillier & Cameron, 1975a,b). This apparent facilitatory effect of immunization against oestradiol may be explained in terms of the affinities of the antibodies and oestradiol receptors in the tumour. The affinities of both antibodies (Hillier et al. 1975a,b) and the high affinity oestradiol binding protein in tumour tissue (McGuire, Chamness, Costlow & Shepherd, 1974) are of the same order of magnitude. Thus, the tumour receptors could compete successfully for antibody-bound oestradiol. Moreover, the high circulating levels of oestradiol in the immunized animals could well result in a greater uptake of oestradiol by the tumours (although this was not determined by Hillier & Cameron, 1975a,b), hence the enhancement of the incidence and growth of tumours.

A further limitation of immunological studies using active immunization against steroids could arise from the saturation of antibody binding sites due to the increased gonadal steroid secretion, thus providing a pool of biologically active steroid which may then exert its normal feedback action on gonadotrophin secretion. This hypothesis is illustrated in a study by Thorneycroft, Thorneycroft, Scaramuzzi & Blake (1975) who showed that normal plasma LH levels could occur in the presence of significant antibody titres and elevated plasma testosterone concentrations in long-term testosterone-immunized male rabbits. This limitation, however, can be overcome to a large extent by booster immunizations prior to the period of study thereby resulting in increased antibody titres.

Of prime importance in immunological studies is the specificity/

specificity of the antibodies. Unless this parameter is adequately assessed, the role of a particular steroid in the hypothalamic-pituitary-gonadal feedback systems cannot be defined. All the active immunization studies cited, where antibody specificity was assessed, used the radioimmunoassay method for this determination. As mentioned earlier, antibody specificity in the radioimmunoassay context compares the displacement of the antibody-bound radioactively labelled hormone by unlabelled hormones at relatively high antiserum dilutions. Specificities determined by this technique, therefore, do not necessarily reflect the in vivo situation.

Nieschlag et al. (1974) examined the effects of immunization against a range of steroid-protein conjugates in the male rabbit. Using equilibrium dialysis, it was demonstrated that the binding of steroids other than the steroid against which the animal was immunized could also be increased. This finding does not, however, necessarily reflect the presence of high-affinity antibodies since the measurement of steroid binding at low dilutions of plasma by equilibrium dialysis could also include low affinity antibody- and albumin-steroid inter-actions (Sandberg, Slaunwhite & Antoniades, 1957; Wu, Motohashi, Abdel-Rahman, Flickinger & Mikhail, 1976).

It is apparent that there are several limitations in the use of active immunization against steroid-protein conjugates to determine the role of gonadal steroids in the feedback mechanisms controlling gonadotrophin secretion. These limitations will be discussed in greater detail in the course of the present study on the effects of active immunization against ovarian steroids on gonadotrophin secretion in the adult non-pregnant ewe.

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## CHAPTER 2

### THE AIMS OF THE PRESENT INVESTIGATION

The endocrinology of the ewe has been the subject of considerable research. The secretion of LH (e.g. Niswender, Roche, Foster & Midgley, 1968; Goding, Catt, Brown, Kaltenbach, Cumming & Mole, 1969; Scaramuzzi, Caldwell & Moor, 1970b; Yuthasastrakosol, Palmer & Howland, 1975) and, to a lesser extent, FSH (Salamonsen, Jonas, Burger, Buckmaster, Chamley, Cumming, Findlay & Goding, 1973; Cunningham, Symons & Saba, 1975) have been described. Ovarian secretion in the ewe has been somewhat more difficult to evaluate due to the low concentration of steroids (apart from progesterone) present in the peripheral circulation. Early studies utilized the somewhat stressful technique of ovarian cannulation to determine the presence of steroids in ovarian venous blood (Short, McDonald & Rowson, 1963; Lindner, Sass & Morris, 1964). An autotransplantation technique enables the transfer of the ovary (Goding, McCracken & Baird, 1967) together with the uterus (Harrison, Heap & Linzell, 1968) to the neck, making the ovarian blood supply more accessible and greatly facilitating the collection of ovarian venous blood. Using this technique, the ovarian secretion rate of several steroids including oestradiol- $17\beta$ , oestrone, androstenedione, testosterone and progesterone have been described (Baird, Goding, Ichikawa & McCracken, 1968b; McCracken, Uno, Goding, Ichikawa & Baird, 1969; Baird, McCracken & Goding, 1973).

Using the classical techniques of ovariectomy and steroid replacement, the positive and negative feedback actions of oestradiol- $17\beta$  on gonadotrophin secretion in the ewe have been demonstrated (Butler, Malven, Willett & Bolt, 1972; Reeves, O'Donnell & Denorscia, 1972; Diekman & Malven, 1973; Davis & Borger/



Borger, 1974). The ability of progesterone to block the positive feedback action of oestradiol on LH secretion has been demonstrated (Scaramuzzi, Tillson, Thorneycroft & Caldwell, 1971). Recently, it has been suggested that progesterone may also exert a negative feedback effect on LH secretion (Foster & Karsch, 1976; Karsch, Foster, Legan & Hauger, 1976). The physiological functions, if any, of oestrone and the androgens, however, have not been elucidated.

The classical techniques of castration and steroid replacement have contributed greatly to the present understanding of the steroid feedback control of gonadotrophin secretion. These techniques, however, cannot take into account interactions between steroids which may serve to modify the secretion of gonadotrophins in the intact animal. Active immunization against steroid-protein conjugates offers a situation whereby, in theory, individual components of the gonadal steroid spectrum may be neutralized. The inhibition of an individual steroid might well alter gonadotrophin secretion thus suggesting a role in the feedback control systems which may not be apparent in the castrate animal (i.e. in the absence of other steroids).

The present study set out to examine the effects of active immunization with four steroid-protein conjugates (fig.2.1), androstenedione-11 $\alpha$ -hemisuccinyl-BSA, testosterone-3-(O-carboxymethyl)oxime-BSA, 17 $\beta$ -oestradiol-6-(O-carboxymethyl)oxime-BSA and oestrone-6-(O-carboxymethyl)oxime-BSA on gonadotrophin secretion in the non-pregnant ewe.

The primary aim of the present study was an examination of the gonadotrophin concentrations in the immunized animals thereby providing a clue to the functions of these steroids in the/



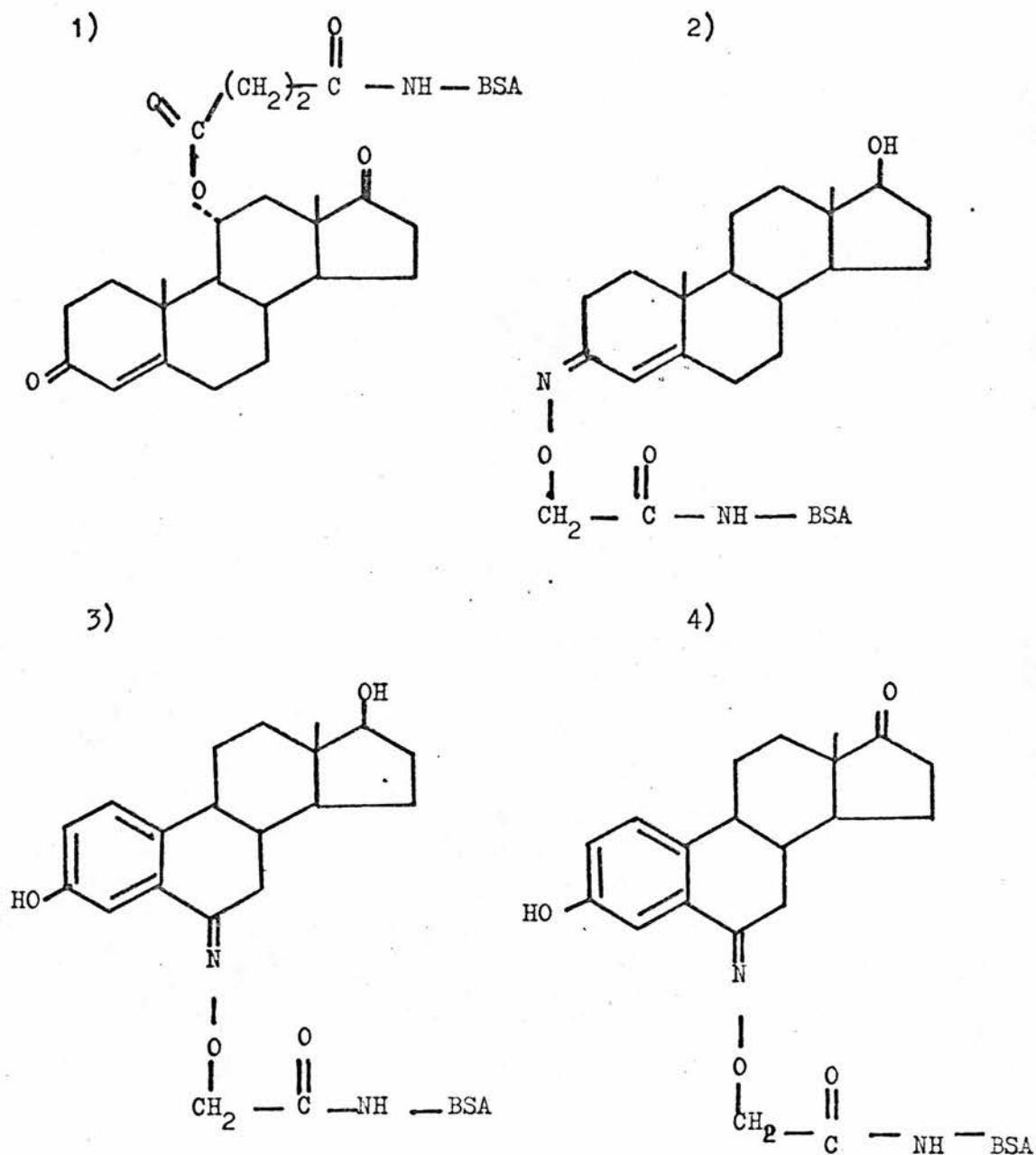


Figure 2.1.

The steroid-protein conjugates.

- 1) Androstenedione-11 $\alpha$ -hemisuccinyl-bovine serum albumin.
- 2) Testosterone-6-(0-carboxymethyl)oxime-bovine serum albumin.
- 3) 17 $\beta$ -oestradiol-6-(0-carboxymethyl)oxime-bovine serum albumin.
- 4) Oestrone-6-(0-carboxymethyl)oxime-bovine serum albumin.

the hypothalamic-pituitary-ovarian axis of the ewe. During anoestrus, the concentrations of both FSH and LH were determined during a twelve hour period. In animals immunized against androstenedione, oestrone and testosterone, the gonadotrophic response to exogenous oestradiol was determined in order to evaluate any modification in the negative and positive feedback actions of this steroid. Gonadotrophin secretion was also examined on four days during the oestrous cycle.

The effects of active immunization against ovarian steroids would not be complete without an examination of ovarian morphology and steroid secretion. The secondary aim of the present study, therefore, was to determine the alterations in ovarian morphology and steroid secretion resulting from the antibodies themselves and/or abnormal gonadotrophin secretion.

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CHAPTER 3.

MATERIALS AND METHODS.

### 3.1. LABORATORY METHODS

#### 3.1.1. RADIOIMMUNOASSAY OF OVINE LUTEINIZING HORMONE.

The measurement of LH in the plasma and pituitary of the sheep by radioimmunoassay has been well established (Geschwind & Dewey, 1968; Niswender et al. 1968; Goding et al. 1969; Niswender, Reichert, Midgley & Nalbandov, 1969; Wheatley & Radford, 1969; Reeves, Arimura & Schally, 1970; Scaramuzzi et al. 1970b). The double antibody radioimmunoassay used in the present study was developed using an antiserum to ovine LH raised in the rabbit and an  $^{125}\text{I}$ -labelled purified ovine LH preparation.

##### (a) Reagents.

The reagents and their suppliers are listed below.

Radioactive sodium iodide ( $\text{Na } ^{125}\text{I}$ ) - The Radiochemical Centre, Amersham.

Bovine serum albumin (Fraction V) (BSA) - Sigma Chemical Co.

Donkey anti-rabbit serum and rabbit serum - Wellcome Reagents Ltd.

Thiomersalate - Hopkin & Williams Ltd.

Tween 20 - Koch-Light Laboratories.

Sephadex G-50 - Pharmacia Fine Chemicals.

Cellulose CF-11 - Whatman Pharmaceuticals.

The following reagents were all Analar Grade from British Drug House: Sodium Dihydrogen orthophosphate ( $\text{NaH}_2\text{PO}_4$ , dihydrate); disodium hydrogen orthophosphate ( $\text{Na}_2\text{HPO}_4$ , anhydrous); sodium chloride; sodium metabisulphite; potassium chloride; Chloramine-T; sodium hydroxide; ethylenediamine tetra-acetic acid (disodium salt, EDTA); Diethyl-barbituric acid.

0.01M phosphate buffered saline (pH 7.8) (PBS) was prepared with 91.6 ml of 0.4M  $\text{Na}_2\text{HPO}_4$  (56.8g/l.) and 8.4ml of 0.4M  $\text{NaH}_2\text{PO}_4$ /

$\text{NaH}_2\text{PO}_4$  (62.4g/l) in 3.9 litres of distilled water containing 36g of sodium chloride and 0.4g of thiomersalate. This buffer formed the basis of other buffers used in the assay and cited below. Buffers containing protein were stored at  $4^\circ\text{C}$ . Buffers containing EDTA were adjusted to pH 7.8 with sodium hydroxide before the addition of protein.

0.12M barbitone buffer (pH 8.5) used in the iodination procedure was prepared by dissolving 442g of diethylbarbituric acid in 18 litres of distilled water. After the addition of 1 litre of 1.9N sodium hydroxide (76g/l), the solution was stirred for 2 hours. A further 1 litre of distilled water was added and the solution stirred for 24 hours.

The assay was performed in disposable polystyrene tubes (10 x 65 mm) (Sarstedt).

The following preparations of ovine pituitary hormones were obtained from the National Institute for Arthritis, Metabolism and Digestive Diseases, Bethesda, U.S.A. (NIAMDD).

Ovine LH (NIH-LH-S12 and S14); Ovine FSH (NIH-FSH-S10); ovine growth hormone (NIH-GH-S11); ovine thyroid-stimulating hormone (NIH-TSH-S8); ovine prolactin (NIH-P-S8).

(b) Iodination of ovine LH.

Initial studies were performed using iodinated Q-3223, a purified ovine pituitary LH preparation with a stated biological potency  $2.14 \times \text{NIH-LH-S1}$  by the ovarian ascorbic acid depletion bioassay (prepared by Dr. H. Papkoff, University of California, San Francisco, U.S.A.). On exhaustion of its supply, a second preparation, LER-1374A (obtained from NIAMDD) was iodinated and used without alteration in the characteristics of the standard curve.

Two methods of iodination were performed, both using the Chloramine-T method of Greenwood, Hunter and Glover (1963) and differing in the method of separation of the iodinated hormone from the unreacted (free) radioactive iodine.

(i) Iodination of ovine LH with separation by gel filtration.

5 $\mu$ g of ovine LH (in 10 $\mu$ l of PBS) was placed in a polystyrene tube and the following reagents added in sequence:

25 $\mu$ l of 0.4M PBS; 20 $\mu$ l of Na<sup>125</sup>I (2mCi); 10 $\mu$ l of chloramine-T (5mg/ml in PBS).

After 30 seconds, the reaction was stopped by the addition of 0.75ml of sodium metabisulphite (0.16mg/ml in PBS) followed by 0.2ml of potassium iodide (20mg/ml in PBS).

The solution was mixed and the total radioactivity determined by measuring the number of counts in the reaction tube during a 10-second period with a ratemeter (NE Scaler-Ratemeter SR3) calibrated to give 14,000 counts/10 seconds = 1mCi.

The reaction mixture was transferred to a column containing 1g of Sephadex G-50 (125 x 10 mm) previously equilibrated with PBS containing 2% BSA. The iodinated hormone was separated from free <sup>125</sup>I by elution with PBS containing 1% BSA and 0.01M EDTA. One millilitre fractions were collected and the radioactivity measured. Iodinated LH was eluted in fractions 4 - 7 and free <sup>125</sup>I eluted in fractions 9 - 16 (fig. 3.1). The iodinated LH was stored at 4°C and used within 14 days.

The approximate specific activity of the iodinated hormone (<sup>125</sup>I-LH) was determined by the following equations:

Amount of radioactivity transferred to the column ( $\mu$ Ci):

$$Z = \frac{TC - RT}{14} \quad \text{where TC} = \text{total counts.}$$

RT = counts remaining in the reaction tube after the transfer of the solution to the column.

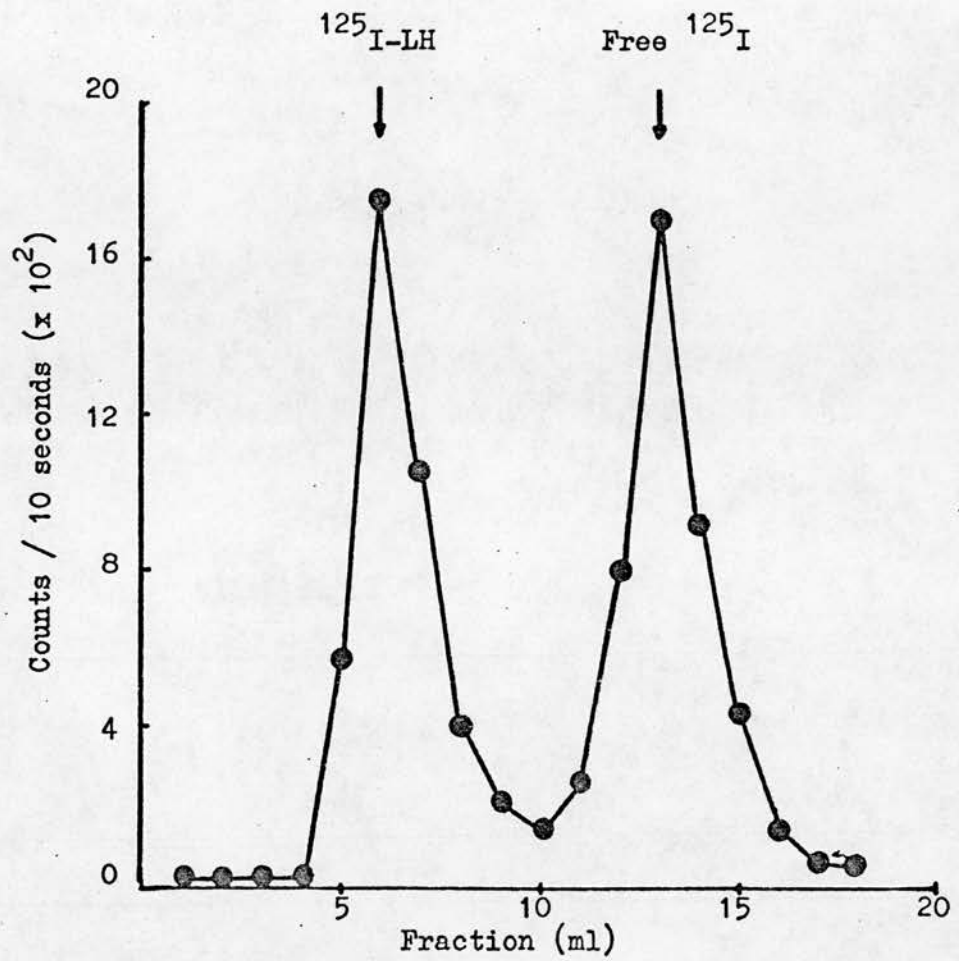


Figure 3.1. The elution of  $^{125}\text{I}$ -ovine LH with Sephadex G-50

On the assumption that RT is due to  $^{125}\text{I}$ -LH, the amount of hormone transferred to the column ( $\mu\text{g}$ ) was determined by:

$$Y = 5 - (5 \times \frac{RT}{TC}).$$

The percentage of radioactivity incorporated into the protein:

$$A = \frac{B}{TC} \times 100 \quad \text{where } B = \text{sum of all the counts in the fractions containing protein.}$$

$$\text{Specific activity } (\mu\text{Ci}/\mu\text{g}) = \frac{Z \times A}{100 \times Y}$$

Specific activities of  $^{125}\text{I}$ -LH obtained by this method ranged from 40.1 - 158  $\mu\text{Ci}/\mu\text{g}$ . Dilution of the iodinated hormone to  $\text{ng}/\text{ml}$  resulted in 25-35% binding by the antiserum under the assay conditions described below.

(ii) Iodination of ovine LH with separation by cellulose chromatography.

The removal of damaged  $^{125}\text{I}$ -labelled hormones by cellulose chromatography after separation of the  $^{125}\text{I}$ -hormone from free  $^{125}\text{I}$  by gel filtration has been described (Goding et al. 1969; Hunter, 1969). In the present studies, the damaged hormone was removed together with the free  $^{125}\text{I}$  by cellulose chromatography of the reaction mixture without prior purification.

2.5 $\mu\text{g}$  of ovine LH in 25 $\mu\text{l}$  of 0.5M PBS was placed in a polystyrene tube and the following reagents added in sequence:

7.5 $\mu\text{l}$  of  $\text{Na } ^{125}\text{I}$  (750 $\mu\text{Ci}$ ) and 10 $\mu\text{l}$  of chloramine-T (5mg/ml in 0.05M PBS). After 15 seconds the reaction was stopped by the addition of 0.5ml of sodium metabisulphite (0.16mg/ml in 0.05M PBS) followed by 0.5ml of potassium iodide (20mg/ml in 0.12M barbitone buffer). The mixture was shaken for 30 seconds and the total counts determined as described above.

The/



The solution was transferred to a column containing cellulose CF-11 (120 x 60 mm) previously equilibrated with barbitone buffer. The column was eluted with 30ml of barbitone buffer and the free  $^{125}\text{I}$  together with damaged  $^{125}\text{I}$ -hormone collected in 5ml fractions. The column was then eluted with 20ml of barbitone buffer containing 5% BSA and 1ml fractions were collected.  $^{125}\text{I}$ -LH was eluted in fractions 1 - 4 (fig. 3.2). It was not possible to determine the specific activity of the  $^{125}\text{I}$ -labelled hormone produced by this method since an unknown amount of damaged hormone was eluted with the free  $^{125}\text{I}$  iodine. However, dilution of the  $^{125}\text{I}$ -LH to give 10,000 cpm/0.1ml gave 35 - 40% binding with the antiserum under the assay conditions described below and standard curves with similar characteristics to the those constructed with  $^{125}\text{I}$ -LH obtained after gel filtration.

The principal advantages of the second iodination procedure lay in the increased binding by the antiserum and the extension of the useful life of the labelled hormone to 4 weeks.

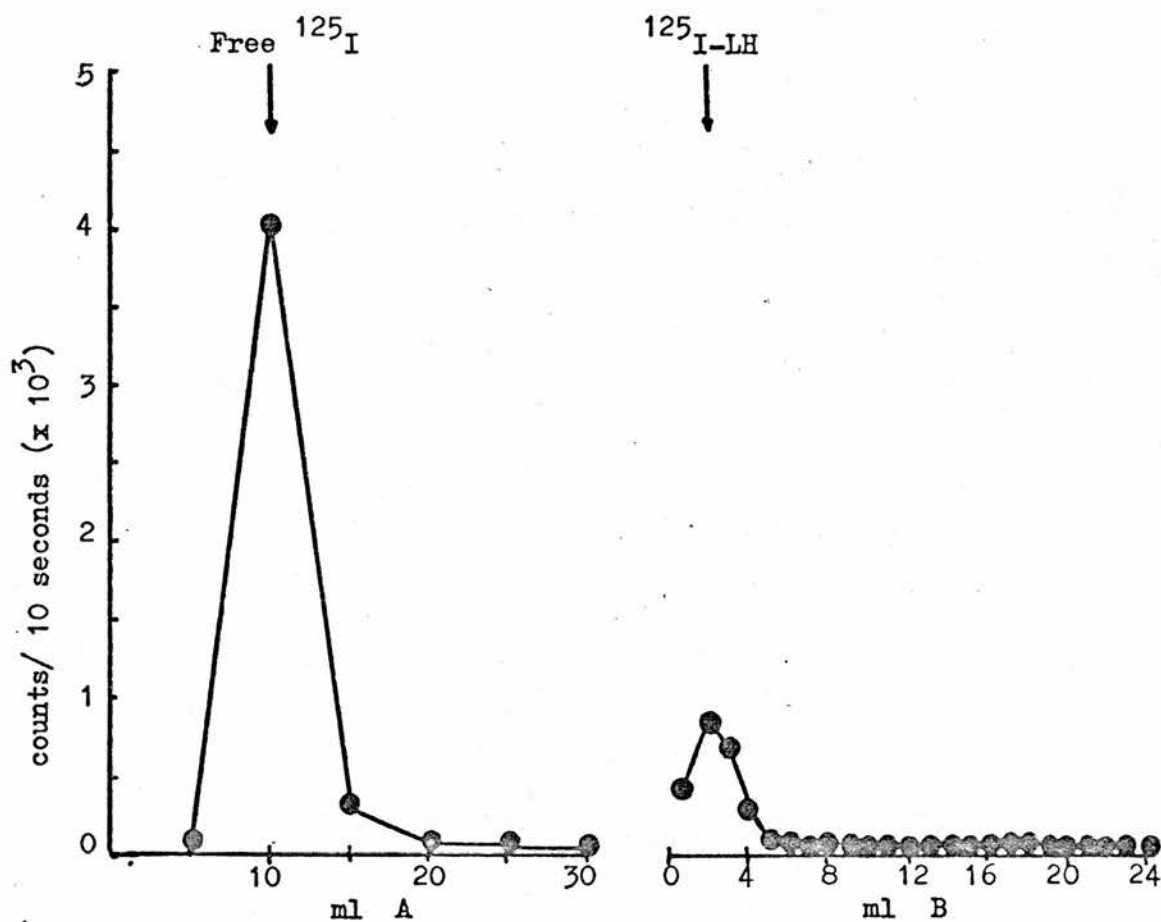
(c) Production of the antiserum to ovine LH.

Antisera to ovine LH were raised in 6 rabbits by Dr. R.J. Scaramuzzi, M.R.C. Reproductive Biology Unit, as previously described (Scaramuzzi et al. 1970b). In brief, each rabbit received three injections into the toepads of 1 or 2mg of antigen (NIH-LH-S11 or S12) emulsified in equal volumes of saline and Freund's complete adjuvant. Blood samples were obtained at intervals from the marginal ear vein.

Preliminary testing of the antisera from the rabbits for possible use in a radioimmunoassay of ovine LH was performed by the assessment of the titre of antibodies capable of binding  $^{125}\text{I}$ -LH.

The/

Figure 3.2. The elution of  $^{125}\text{I}$ -ovine LH with cellulose CF-11



A = 0.12M Barbitone buffer - 5ml fractions collected.

B = 2% BSA in 0.12M Barbitone buffer - 1ml fractions.

The titre was defined as the initial dilution of serum which bound 50% of 0.1ng of  $^{125}\text{I}$ -LH under the assay conditions described below. The titres present in the sera are shown in Table 3.1. Appreciable binding was observed in all sera with maximum binding occurring in sera obtained 125 and 104 days after the primary immunization in rabbits 1-5 and rabbit 115 respectively. Several antisera showed titres in excess of 1:50,000. One antiserum, R115-104 was chosen arbitrarily and further studies were performed with this antiserum.

(d) The radioimmunoassay procedure.

The assay procedure was based on the radioimmunoassay of rabbit LH described by Scaramuzzi, Blake, Papkoff, Hilliard and Sawyer (1972).

The assay consisted of duplicate aliquots of plasma and a standard curve of 0.1ml aliquots of known amounts of ovine LH (NIH-LH-S14). The volume was adjusted to 0.7ml with PBS containing 0.2% BSA (the diluent buffer). The binding of  $^{125}\text{I}$ -LH in the absence of unlabelled LH (zero standard) was determined in duplicate tubes containing 0.7ml of diluent buffer. All tubes received 0.1ml of the anti-LH serum (diluted 1:200,000 in diluent) and were incubated overnight at 4°C. After the addition of 0.1ml of  $^{125}\text{I}$ -LH (diluted to give 10,000cpm/0.1ml in PBS containing 0.01M EDTA, 0.2% BSA and 1/300 rabbit serum), the tubes were incubated for 3 days at 4°C. The separation of free  $^{125}\text{I}$ -LH from the antibody-bound hormone was performed by the addition of 0.1ml of a 1/60 dilution of donkey anti-rabbit serum. After incubation for 12 hours, all tubes received 1ml of PBS containing 1% Tween 20 and were centrifuged at 3000 rpm for 30 minutes at 4°C. The supernatant/

TABLE 3.1

Titres of LH antibodies in rabbits immunized against ovine LH<sup>a</sup>

<u>Rabbit No.</u>	<u>Days after the primary immunization</u>	<u>Titre<sup>b</sup></u>
1	48	1:4600
	60	1:2100
	116	1:52000
	125	1:64000
2	38	1:2100
	48	1:2400
	60	1:6600
	125	1:66000
3	38	1:1500
	44	1:1500
	48	1:1700
	60	1:3000
	125	1:74000
4	38	1:2200
	48	1:2400
	60	1:10300
	116	1:46000
	125	1:35000
5	48	1:7000
	60	1:19000
	116	1:45000
	125	1:45000
115	53 <sup>c</sup>	1:18000
	104	1:64000

<sup>a</sup> Rabbit numbers 1-5 were immunized against 2mg NIH-LH-S12, Rabbit number 115 was immunized against NIH-LH-S11.

<sup>b</sup> Titre = initial dilution of serum which bound 50% of 0.1g <sup>125</sup>I-LH in a total volume of 1ml.

<sup>c</sup> Antiserum no. 115-53 has been described elsewhere (Scaramuzzi et al. 1970b).

supernatant was decanted and the radioactivity remaining in the precipitate adhering to the bottom of the tube measured with an automatic gama spectrometer (Wallac Gamma Counter GTL 300-500).

The non-specific binding (NSB) of  $^{125}\text{I}$ -LH in the absence of antibodies to LH was determined in duplicate tubes containing 0.8ml of diluent and 0.1ml of  $^{125}\text{I}$ -LH and treated as described above without the addition of the LH antiserum. The total radioactivity added to each tube was determined in two tubes containing 0.1ml of  $^{125}\text{I}$ -LH.

(e) Calculations.

The line of best-fit was constructed for the standard curve (fig. 3.3) after log-logit transformation of the data (Midgley, 1969) using a programme compiled by R.M. Sharpe (M.R.C. Reproductive Biology Unit) for the Hewlett Packard 9812A Calculator.

In brief, the Y-axis represents the logit transformation,

$$\log \frac{e^y}{(1 - y)} \quad \text{where } y = \frac{\text{cpm standard} - \text{NSB}}{\text{cpm zero standard} - \text{NSB}}$$

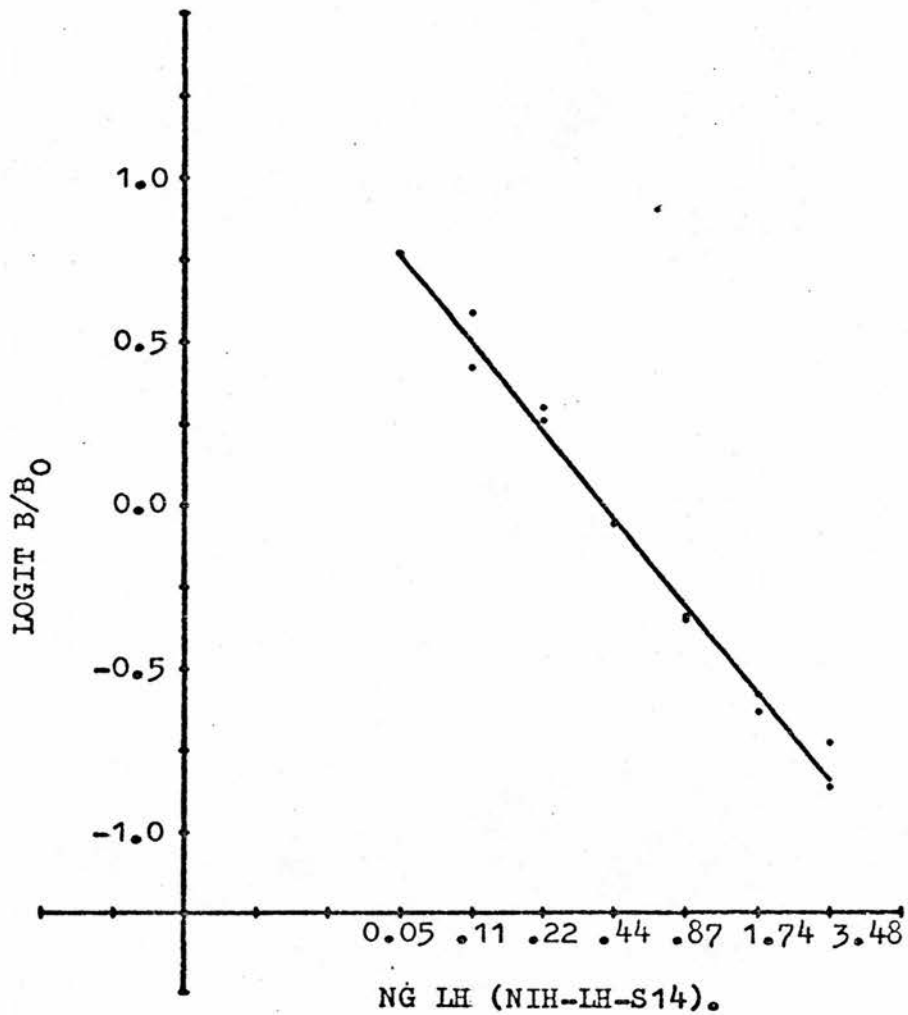
and the X-axis represents the nanogram values of the standards plotted on a log scale. The line of best-fit was calculated by the method of least squares. Points were omitted if their calculated value differed by more than 30% of their theoretical value and a new line of best-fit calculated. This process of elimination was repeated to exclude points whose values differed by more than 20% and 10% of their theoretical values. The line drawn represents the line of best-fit after the three elimination calculations had been performed (fig. 3.3).

The radioactivity of the unknowns was transformed to logits and the concentration of LH determined from the standard curve. The results are expressed in terms of ng of NIH-LH-S14/ml of plasma.

(f)/

FIGURE 3.3

The LH standard curve.



Slope = -0.8920; Y-intercept = 2.3175.

Limit of sensitivity = 53pg

B = cpm standard - NSB.

B<sub>0</sub> = cpm zero standard - NSB.

(f) Validation of the assay.(i) The standard curve.

The standard curve (fig. 3.3) consisted of a straight line drawn through 7 points with concentrations of LH ranging from 0.05 to 3.4ng. The mean ( $\pm$  s.e.m.) slope and Y-intercept of the standard curves of 24 assays was  $-0.91 \pm 0.05$  and  $2.46 \pm 0.12$  respectively.

The limit of sensitivity of the assay was initially determined by the concentration of LH which depressed the percentage binding of  $^{125}\text{I}$ -LH by 2 standard deviations below the mean of 9 zero standards, and subsequently, by the amount of LH which depressed the binding of the zero standards by 10%. The upper limit of the standard curve ranged from 0.05 to 0.075ng. The lower limit of the standard curve was defined as the concentration of LH which depressed the binding of the zero standards by 90%. In the routine assay, samples which showed concentrations greater than 3.4ng/tube were re-assayed at a higher dilution.

(ii) Specificity.

The specificity of the antiserum was evaluated by comparing the dose-response curves of NIH-LH-S14 and other ovine pituitary hormones. The cross reaction was determined from the following formula (Abraham, et al. 1970):

$$\text{Cross reaction (\%)} = \frac{A}{B} \times 100$$

where A = the mass of LH required to displace 50% of the bound  $^{125}\text{I}$ -LH; and B = the mass of the test hormone required to displace 50% of the bound  $^{125}\text{I}$ -LH in the absence of unlabelled LH.

The cross reactions with some ovine pituitary hormones are shown in Table 3.2. The highest cross reactions occurred with TSH (9%), FSH (4%) and growth hormone (3%). Although the cross reaction/



TABLE 3.2

The specificity of the LH antiserum (R115-104).

<u>Hormone tested</u>	<u>Range tested</u> (ng)	<u>Cross reaction</u> (50% level)
Ovine LH (NIH-LH-S12)	0.01 - 40	100
Ovine LH (NIH-LH-S14)	0.01 - 40	100
Ovine LH (Papkoff, Q3-223)	0.01 - 40	100
Ovine FSH (NIH-FSH-S10)	0.01 - 40	3.7
Ovine growth hormone (NIH-GH-S11)	0.01 - 40	3.1
Ovine TSH (NIH-TSH-S8)	0.01 - 40	9
Ovine Prolactin (NIH-P-S8)	0.1 - 400	0.001

Cross reaction (%) =  $\frac{A}{B} \times 100$ , where A = mass of LH required to displace 50% of bound  $^{125}\text{I}$ -ovine LH; B = mass of test compound required to displace 50% of bound  $^{125}\text{I}$ -ovine LH.

reaction with TSH is higher than the stated biologically active LH content of the TSH preparation (less than 1%), there is some evidence to suggest that antisera with a high immunological cross reaction with pituitary TSH preparations do not show elevated levels of LH after the release of TSH by thyrotropin releasing hormone (Carr & Land, 1975; Lincoln & Peet, unpublished observations). It is possible, therefore that the high immunological cross reaction with a pituitary TSH preparation may not necessarily confer a high degree of cross reactivity with biologically active TSH in plasma. This approach cannot be used to test the cross reactivity with FSH since a separate FSHRH has so far not been isolated.

(iii) Recovery of exogenous LH from plasma.

The accuracy of the assay was evaluated by the recovery of exogenous LH from non-reactive plasma (human). Exogenous LH (0.05-3.4ng) was added to several volumes of human plasma (0.1-0.4ml). A plot of the concentration of LH measured and the amount of LH added yielded a straight line with a mean regression of  $y = 0.87x + 0.035$ , where  $y$  = ng LH measured and  $x$  = ng LH added. The recovery of LH from plasma was not significantly different from the recovery of LH from buffer.

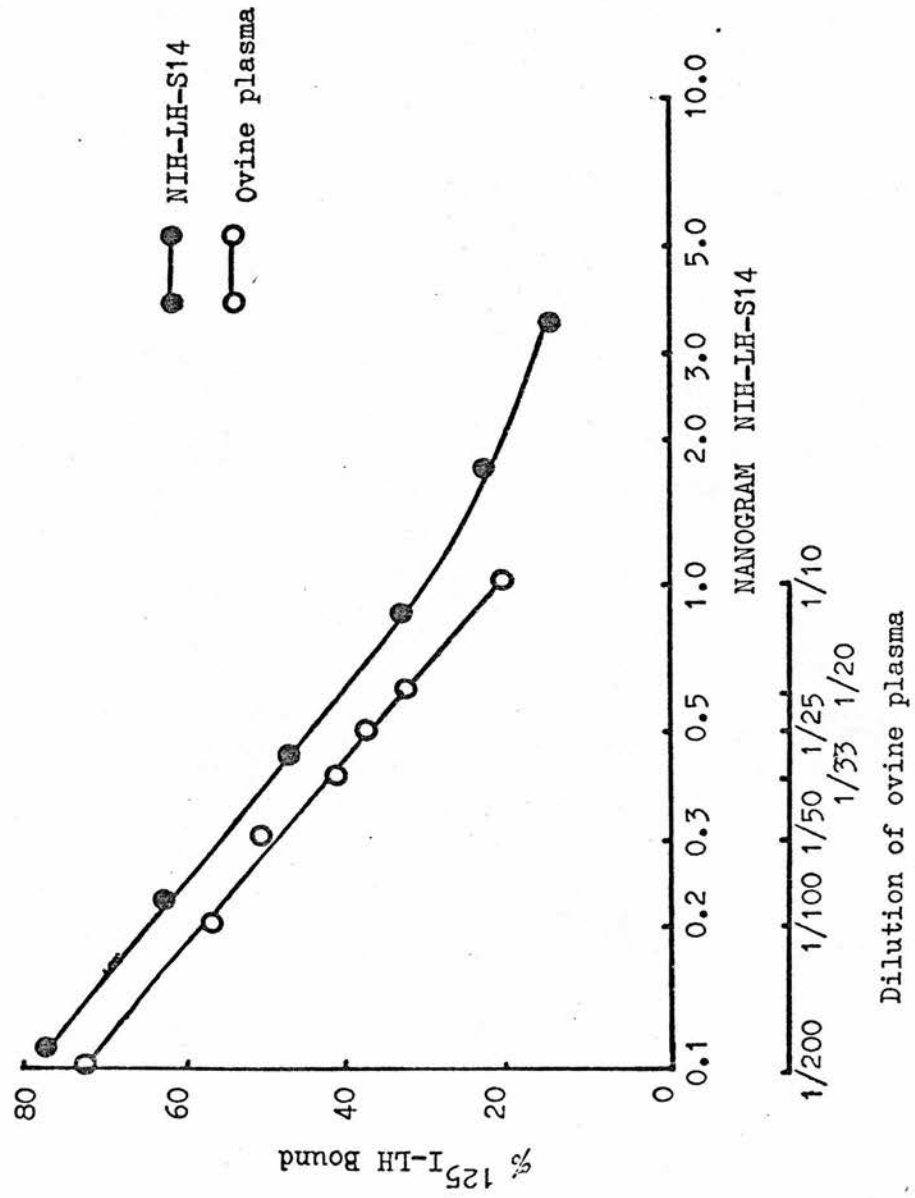
(iv) Parallelism of ovine plasma with the standard curve.

No evidence of non-parallelism was found between the serial dilution of ovine plasma with a high LH content and the standard curve (fig.3.4) when tested by 2-factor analysis of variance.

(v) Precision.

The within-assay variation was determined by the repeated assay of an ovine plasma sample within an assay. The sample gave a mean concentration of LH ( $\pm$  S.D.) of  $20.46 \pm 0.81$  ng/ml ( $n=12$ ) with/

Figure 3.4 The dose-response curves of LH and a serial dilution of ovine plasma.



with a coefficient of variation of 8.1%.

The inter-assay variation of the assay was assessed by the inclusion of pooled ovine plasma samples in each assay. The mean levels ( $\pm$  S.D.) of three plasma samples over 24 assays was  $22.90 \pm 1.86$  ng/ml,  $11.41 \pm 1.27$  ng/ml and  $1.40 \pm 0.21$  ng/ml with coefficients of variation of 8.4%, 11.2% and 15.0% respectively.

(vi) The concentration of LH during the oestrous cycle of the ewe.

The concentration of LH was determined in the plasma from daily jugular venous blood samples taken throughout two oestrous cycles from 5 ewes (kindly supplied by Dr. R.J. Scaramuzzi) (fig. 3.5). The level of LH was low throughout the cycle except on the day of oestrus when a peak of LH was observed in 5 of the 10 samples taken at oestrus.

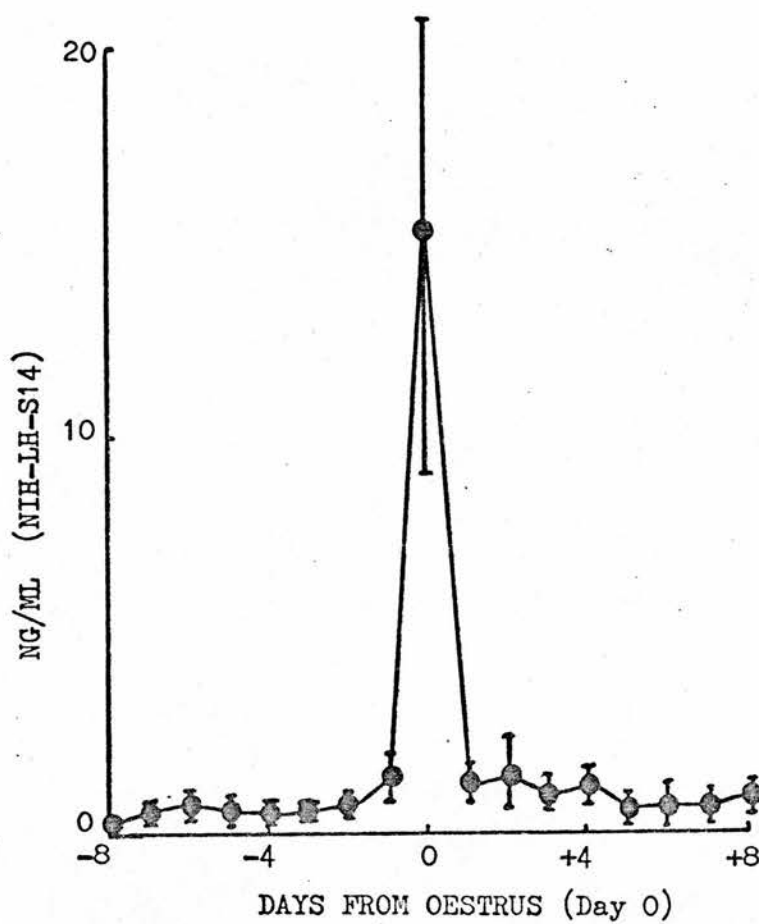
The concentrations of LH during the oestrus cycle of the ewe determined using the radioimmunoassay developed in this laboratory are similar to those reported elsewhere (e.g. Geschwind & Dewey, 1968; Niswender et al. 1968). These results suggest that the radioimmunoassay for ovine LH described above is sufficiently sensitive and specific for the determination of LH in the plasma of the ewe.

3.1.2. THE RADIOIMMUNOASSAY OF OVINE FSH.

Considerable difficulty has been encountered in the measurement of FSH in the plasma of sheep by radioimmunoassay. Antisera raised against ovine FSH show a high cross reaction with ovine LH and ovine TSH making an homologous radioimmunoassay with unabsorbed antisera essentially invalid at the present time - only one such assay has been described (Kragt & Cons, 1972). Attempts to reduce the cross reactions by absorption of the antiserum with ovine TSH and LH have been largely unsuccessful (Bailly du Bois, Kerdelhue, /

Figure 3.5

The concentration of LH during the oestrous cycle of the ewe.



Each point represents the mean of daily samples taken from 5 ewes throughout 3 oestrous cycles. The vertical bars represent the mean  $\pm$  S.E.

The ability of FSH antisera to cross react with FSH from other species has led to the description of several heterologous radioimmunoassays for the determination of FSH in sheep plasma. Heterologous assays using either  $^{125}\text{I}$ -human FSH and a rabbit antiserum to ovine FSH (L'Hermite, Niswender, Reichert & Midgley, 1972) or  $^{125}\text{I}$ -ovine FSH and a rabbit antiserum to human FSH (Salamonsen et al. 1973) have been described. More recently, McNeilly, McNeilly, Walton & Cunningham (1976) reported a sensitive and specific heterologous radioimmunoassay for ovine FSH using an antiserum to human FSH raised in the rabbit and iodinated preparations of either ovine or rat FSH.

A heterologous radioimmunoassay for ovine FSH using an antiserum to ovine FSH raised in the rabbit (Welschen, Osman, Dullaart, De Greef, Uilenbroek & De Jong, 1975) and an iodinated rat FSH preparation was developed by M.J. Peet (M.R.C. Reproductive Biology Unit). This assay has been used in studies on the photoperiodic control of reproduction in the ram (Lincoln, Peet & Cunningham, 1976). The validation of the assay has not been published in detail and will be described briefly.

(a) Reagents.

The reagents and buffers used in the radioimmunoassay of ovine FSH are identical to those described for the assay of ovine LH.

Purified ovine FSH (M4, 14 times the potency of NIH-FSH-S1 by the Steelman-Pohley bioassay) was donated by Professor M. Jutisz, Laboratoires des Hormones Polypeptidiques, C.N.R.S. France.

Purified rat FSH (NIH-RFSH-13) was obtained from NIAMDD.

The/



The anti-ovine FSH serum (FSH-619<sup>II</sup>) raised in the rabbit against NIH-FSH-S9 was generously supplied by Dr. J. Uilenbroek, Medical Faculty, Erasmus University, Rotterdam, Holland. The antiserum was used at an initial dilution of 1:10,000 and at this concentration bound 15-25% of the labelled hormone.

(b) Iodination of ovine and rat FSH.

The iodination procedure essentially followed the Chloramine-T method with cellulose chromatography, as described earlier for ovine LH.

(c) Radioimmunoassay procedure.

The radioimmunoassay protocol was identical to the procedure described for the assay of ovine LH, the main difference lying in the precipitation of the antibody-hormone complex with a 1/24 dilution of donkey anti-rabbit serum.

(d) Validation of the assay.

(i) The homologous radioimmunoassay.

The dose-response curves of ovine FSH, TSH and LH with <sup>125</sup>I-ovine FSH are shown in figure 3.6. The inhibition curves of TSH and LH were not parallel with that of FSH. Furthermore, the response of a serial dilution of plasma from a castrated ram was not parallel with the dose-response curve of FSH. These findings proved the unsuitability of an homologous radioimmunoassay of ovine FSH and no further work with this assay was performed.

(ii) The heterologous radioimmunoassay.

The effect of ovine FSH, TSH, LH, growth hormone and prolactin on the binding of <sup>125</sup>I-rat FSH was examined. The dose-response curves of FSH and LH were parallel (fig. 3.7) with a cross reaction of 8.5% at the 50% binding level. The dose-response curve of TSH was not parallel with that of FSH and showed a cross reaction/



Figure 3.6. The inhibition curves of ovine FSH, TSH, LH and ovine plasma with  $^{125}$ I-ovine FSH.

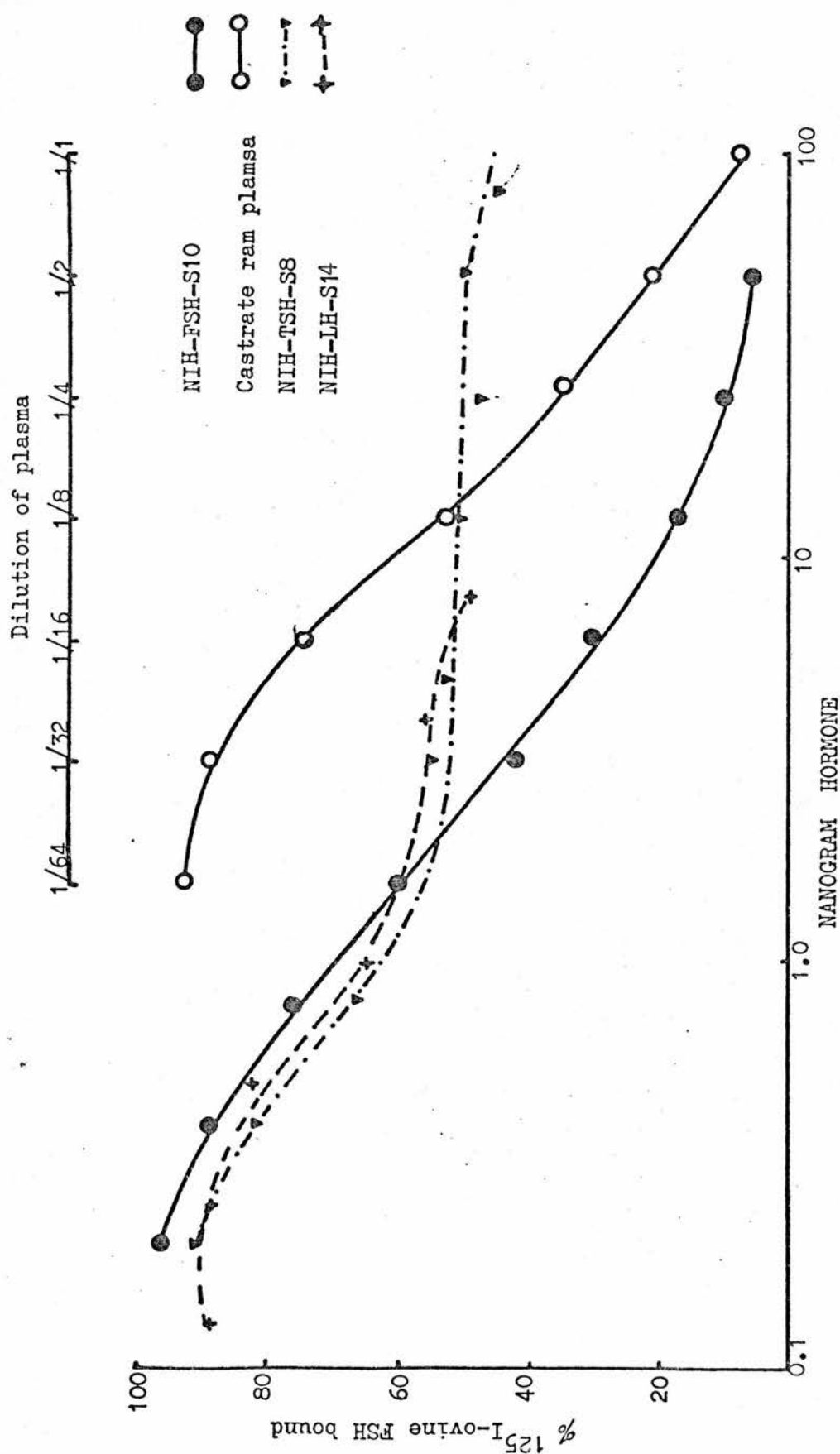
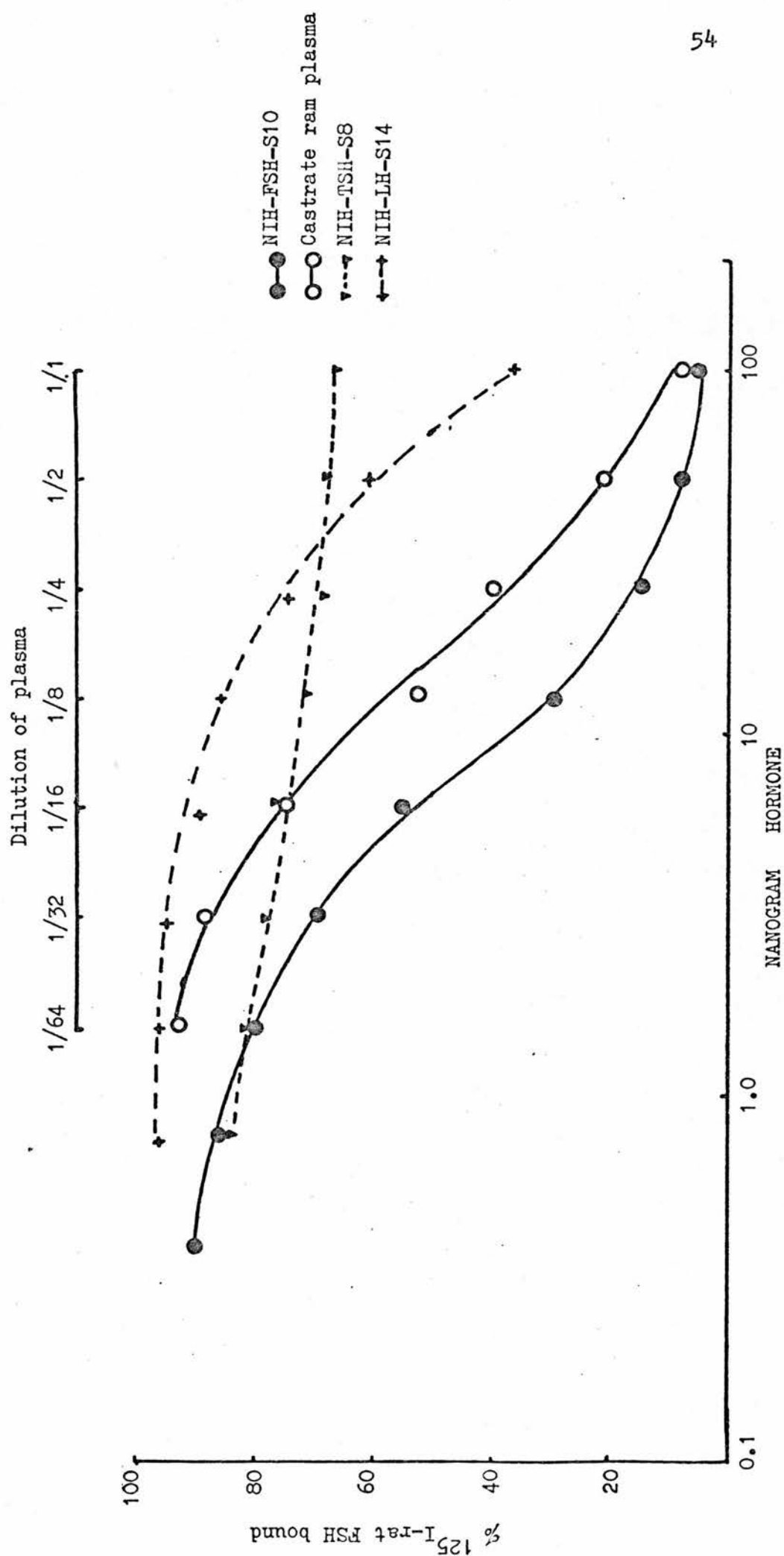


Figure 3.7 The inhibition curves of ovine FSH, TSH, LH and ovine plasma with  $^{125}\text{I}$ -rat FSH.



reaction of less than 0.2%. The shallow slope of the TSH curve indicated the presence of low affinity antibodies (fig.3.7). Ovine growth hormone and prolactin failed to alter significantly the binding of iodinated rat FSH. The inhibition curve of a serial dilution of plasma from a castrated ram was parallel with the standard curve (fig. 3.7).

After log-logit transformation, the standard curve was linear over the range 34.2 - 0.5ng of NIH-FSH-S10 (fig. 3.8). The upper and lower limits of the standard curve were defined as the concentrations of FSH which depressed the binding of  $^{125}\text{I}$ -rat FSH by 10% and 90% respectively. The limit of sensitivity of the assay ranged from 0.8 - 1.8 ng FSH.

The intra-assay variation was assessed by repeated assay of an ovine plasma sample which gave a mean ( $\pm$  S.D.) of  $22.6 \pm 0.32$  ng/ml (n=12) with a coefficient of variation of 4.9%.

The between-assay precision of the assay was evaluated by the inclusion of a plasma sample taken from a ewe during anoestrus in every assay. Over 17 assays, the mean ( $\pm$  S.D.) concentration of FSH was  $21.6 \pm 0.33$  ng/ml with a coefficient of variation of 6.4%.

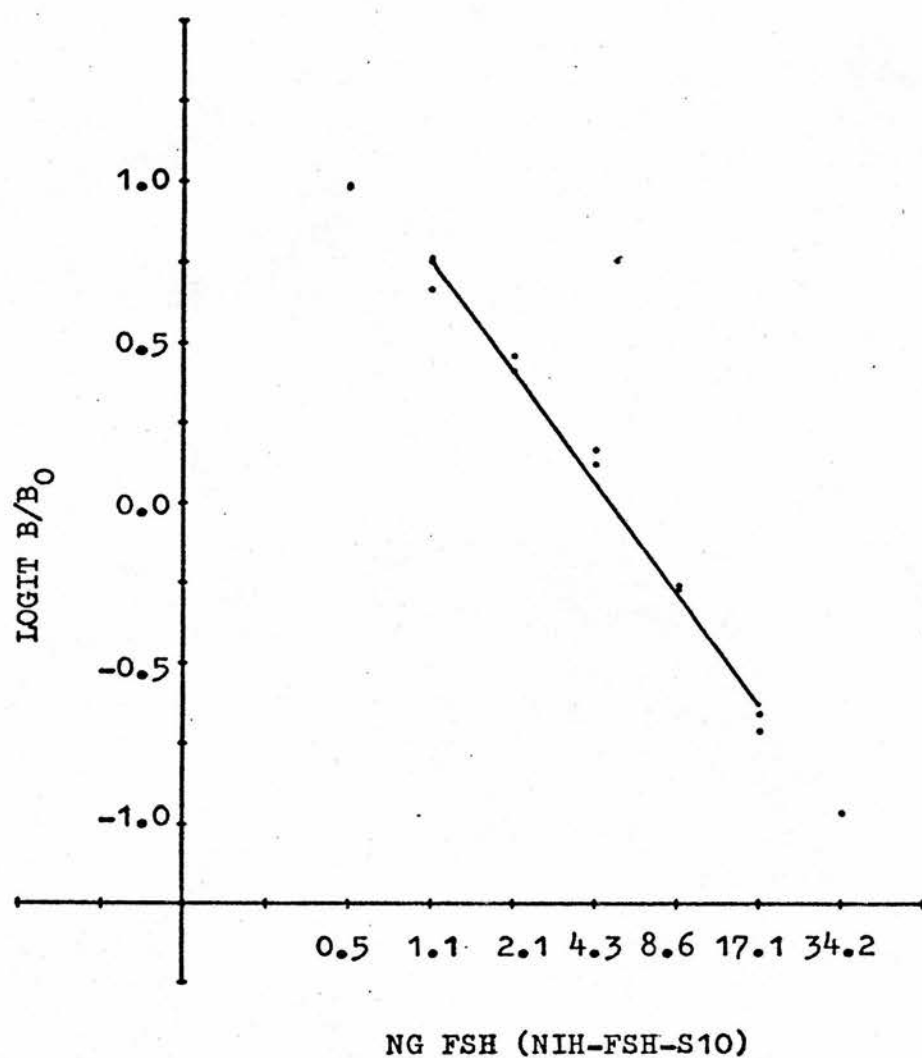
All results are expressed in terms of ng NIH-FSH-S10/ml of plasma.

### 3.1.3. THE PROGESTERONE RADIOIMMUNOASSAY.

The measurement of progesterone in the plasma and ovarian follicular fluid of the ewe was performed using a radioimmunoassay procedure described elsewhere (Scaramuzzi, Corker, Young & Baird, 1975).

The antiserum (361 FB) generously donated by Dr. R.J. Scaramuzzi, was raised in the sheep against progesterone- $11\beta$ -hemisuccinate/

The FSH standard curve.



$B = \text{cpm standard} - \text{NSB.}$

$B_0 = \text{cpm zero standard} - \text{NSB}$

Slope = -1.1563; Y-intercept = 4.2648

Limit of sensitivity = 890pg.

hemisuccinate conjugated to BSA. The major cross reactions of the antiserum were determined by Clarke (1976) and are shown in Table 3.3.

Unlabelled progesterone was obtained from Sigma Chemical Co. and stored in a stock solution of  $10\mu\text{g/ml}$  in ethanol at  $4^{\circ}\text{C}$ .

$1,2,6,7\text{-}^3\text{H}$ -progesterone was obtained from the Radiochemical Centre, Amersham. A stock solution of  $10\mu\text{Ci/ml}$  in ethanol was stored at  $4^{\circ}\text{C}$ . The purity of the stock solution was monitored at intervals by thin-layer chromatography and found to be free of impurities (Clarke, 1976).

0.2ml of the sample (undiluted or diluted plasma or follicular fluid) was extracted with 10 volumes of freshly distilled petroleum ether (b.p.  $40^{\circ} - 60^{\circ}$ ) for 4 minutes with a Multivortex Shaker (Baird & Tatlock) enabling the simultaneous extraction of up to 100 samples.

The organic layer was decanted into glass tubes (10 x 75mm) after freezing the aqueous phase with a mixture of methanol and dry ice. The organic solvent was removed by evaporation under nitrogen at  $50^{\circ}\text{C}$ . The residue was redissolved in 0.2ml of a phosphate-gelatin buffer (PGB) prepared by dissolving 8.6g of disodium hydrogen orthophosphate (anhydrous), 5.4g of sodium dihydrogen orthophosphate (monohydrate), 9g of sodium chloride, 1g of gelatin and 0.001g of thiomersalate in 1 litre of distilled water. The tubes were incubated for 2 hours at room temperature. After mixing, 0.1ml of the extract was removed for the assay of progesterone.

The recovery of progesterone was estimated by the pre-incubation of  $20\mu\text{l}$  of  $^3\text{H}$ -progesterone (2000cpm) in ethanol with the sample for 1 hour at room temperature before extraction. A  $50\mu\text{l}$  aliquot/

The specificity of the progesterone antiserum (361 FB).

<u>Steroid</u>	<u>% Cross reaction</u>
Proges terone	100
5 $\alpha$ -OH-proges terone	8.3
20 $\alpha$ -dihydroproges terone	1.5
11 $\alpha$ -OH-proges terone	4.6
11 $\beta$ -OH-proges terone	6.6
17 $\alpha$ -OH-proges terone	7.1
11-ke to-proges terone	17.1
Pregnenolone	< 0.1
Deoxycorticos terone	21.2
Corticos terone	9.7
Cortisol	< 0.001
Andros tenedione	< 0.1
Oes tradiol-17 $\beta$	< 0.001
Testos terone	< 0.1

Reproduced by permission from Clarke (1976).

aliquot of the extract was removed for the determination of radioactivity.

The assay consisted of 0.1ml aliquots of duplicate extractions of the samples as described above and a standard curve consisting of triplicate tubes containing 0.1ml of 7 dilutions of unlabelled progesterone (31 - 1000pg) in PGB. The binding of  $^3\text{H}$ -progesterone in the absence of unlabelled progesterone (zero standard) was determined in triplicate tubes containing 0.1ml of PGB. All tubes received 0.1ml of the anti-serum (diluted 1:4000 with PGB) followed by 0.1ml of  $^3\text{H}$ -progesterone (diluted to give 50pg/0.1ml with PGB). Also included in the assay were six tubes containing 0.2ml of PGB and 0.1ml of  $^3\text{H}$ -progesterone. Three of these tubes represented an average of the total radioactivity added to all tubes (TC) and the remaining three tubes estimated the non-specific binding of  $^3\text{H}$ -progesterone in the absence of the antiserum (NSB). After mixing, the assay was incubated overnight at 4°C.

The separation of free progesterone from the antibody-bound steroid was performed by charcoal absorption of the free steroid. The "stripping" or removal of progesterone from the steroid-antibody complex by charcoal has been described elsewhere (Clarke, 1976). The assay was maintained at 8°C with an ice-bath and 1ml of a freshly prepared charcoal suspension (Norit A, 1mg/ml in PGB) added to all tubes except the TC tubes which received 1ml of PGB. After 20 minutes, the tubes were centrifuged at 3000rpm for 10 minutes at 8°C and the supernatant decanted into plastic counting vials. After the addition of 10ml of scintillation fluid (12g of 2,5-diphenyloxazole and 900mg p-bis-2-(5-phenyloxazolyl) benzene in 4.5 litres of Toluene containing 33% Triton X-100 (v/v)), the radioactivity was measured with a Packard Tri-Carb liquid/



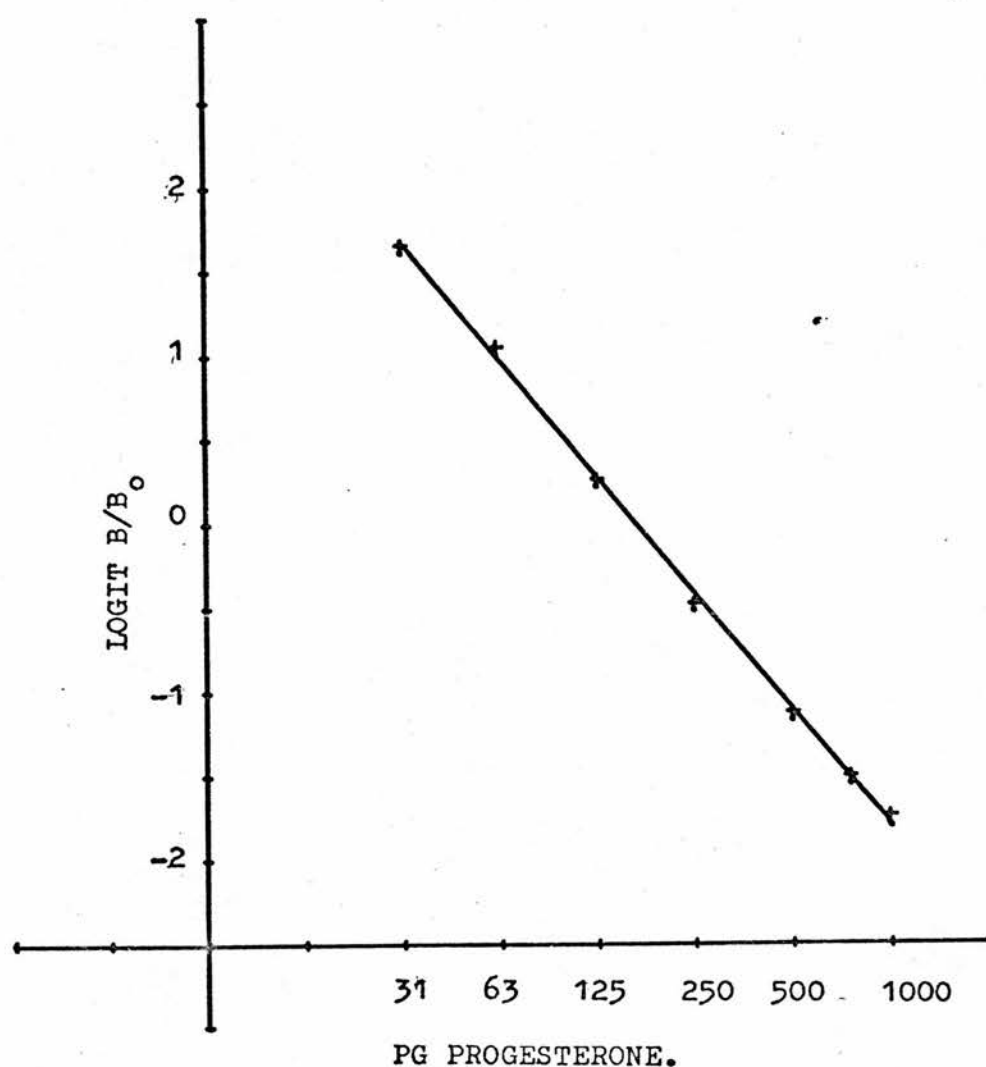
liquid scintillation counter model 3375 with a counting efficiency of about 30%.

The mean recovery of progesterone from 14 assays was 69.3% with a coefficient of variation of 7%. A lower recovery of progesterone (30 - 35%) was obtained from plasma from ewes immunized against androstenedione. The reason for this low recovery is not known and cannot be attributed to the immunization procedure since normal recoveries occurred with plasma from ewes immunized against other steroid-protein conjugates.

After log-logit transformation of the data using the programme previously described, the standard curve was linear over the range 31 to 1000pg (fig. 3.9). The upper and lower limits of the standard curve were determined by the concentrations of progesterone which depressed the binding of the zero standard by 10% and 90% respectively. The limit of sensitivity ranged from 13 to 24pg. The radioactivity of the samples was transformed to logits and the picogram value obtained from the standard curve. After correction for recovery, the results were calculated in terms of ng progesterone/ml of plasma or follicular fluid.

The effect of the organic solvent used in the extraction procedure was assessed by the extraction and assay of 0.2ml of distilled water. Each assay was further validated by the inclusion of a 0.2ml aliquot of a pooled plasma sample from a castrated ram. Assays showing detectable progesterone in either of these two quality controls were repeated.

The inter-assay variation was evaluated by the inclusion in each assay of pooled plasma samples from ovine jugular venous and utero-ovarian venous blood taken during the luteal phase of the oestrous cycle. The mean concentration of progesterone ( $\pm$  s.e.m.) in/

The progesterone standard curve.

Each point represents the mean of triplicate determinations.  
Slope = -0.410; Y-intercept = 2.288;

Limit of sensitivity = 24pg.

B = cpm standard - NSB.

B<sub>0</sub> = cpm zero standard - NSB.

in jugular venous plasma and utero-ovarian venous plasma in 14 assays were, respectively,  $2.2 \pm 0.1$  ng/ml and  $53.9 \pm 2.2$  ng/ml with coefficients of variation of 10.9% and 15.0%.

#### 3.1.4. THE MEASUREMENT OF OESTRADIOL-17 $\beta$ , OESTRONE, TESTOSTERONE AND ANDROSTENEDIONE.

The concentration of these steroids in the plasma and follicular fluid were determined by Dr. P. Van Look, Mr. D.W. Davidson and Mr. I. Swanston (M.R.C. Reproductive Biology Unit).

##### (a) Oestradiol-17 $\beta$ radioimmunoassay.

The concentration of oestradiol-17 $\beta$  in the plasma and follicular fluid of the ewe was determined using the radioimmunoassay of Van Look (1976). The antiserum (A/S D) was raised in the rabbit against 17 $\beta$ -oestradiol-6-(O-carboxymethyl)oxime-BSA (Dean et al. 1971; Exley et al. 1971) and used at an initial dilution of 1:7000 in PGB. The antiserum showed the following cross reactions with phenolic steroids (Van Look, 1976): 6-oxo-oestradiol-17 $\beta$  (106%), oestradiol-17 $\beta$  (100%), oestradiol-17 $\alpha$  (2%), oestriol (1.7%) and oestrone (0.2%).

An aliquot of the plasma or follicular fluid was acidified with 0.5ml of 1N hydrochloric acid and extracted with 2ml of diethyl ether. The ether was removed by evaporation under nitrogen and the residue redissolved in 0.1ml of PGB and assayed for oestradiol as described elsewhere (Van Look, 1976).

##### (b) Oestrone radioimmunoassay.

The radioimmunoassay for oestrone was performed using an antiserum raised in the rabbit against oestrone-6-(O-carboxymethyl)oxime-BSA (Rowe, Cook & Dean, 1973). The antiserum (R4 B9) showed negligible cross reaction (less than 0.1%) with oestradiol-17 $\beta$ , oestriol/

oestriol, androstenedione and testosterone, enabling direct measurement of oestrone without chromatographic separation.

An aliquot of plasma or follicular fluid was extracted with 2ml of diethyl ether. The ether was removed by evaporation under nitrogen and the residue redissolved in 0.1ml of PGB and assayed for oestrone by the addition of 0.1ml of the antiserum and 0.1ml of  $^3\text{H}$ -oestrone and separation of free and bound steroids by charcoal absorption.

(c) The measurement of androstenedione and testosterone.

Androstenedione and testosterone were separated by alumina chromatography and androstenedione determined by a modification of the radioimmunoassay of Baird, Burger, Heavon-Jones & Scaramuzzi (1974) and testosterone by the method of Corker and Davidson (1976).

Aliquots of plasma or follicular fluid were acidified with 0.5ml of 1N hydrochloric acid and extracted with 4ml of hexane:diethyl ether (4:1 v/v). The extract was placed on an alumina column (30 x 5mm) prepared as described elsewhere (McNatty, Baird, Bolton, Chambers, Corker & McLean, 1976) by washing consecutively with 3.2ml of ethanol, 6.4ml of methanol, 4.8ml of methanol:dichloromethane (1:1 v/v) and 4.8ml of dichloromethane. After transfer of the extract, the column was washed with 4ml of hexane:ether (4:1) and 6.4ml of 0.2% ethanol in hexane. Androstenedione was eluted with 3.2ml of 0.2% ethanol in hexane. After washing with a further 3.2ml of 0.2% ethanol in hexane, testosterone was eluted with 3.2ml of 1% ethanol in hexane.

The solvents of both extracts were removed by evaporation under nitrogen, the residues redissolved in 0.5ml of PGB and a 0.1ml aliquot removed for the steroid assays.

The androstenedione antiserum (a gift from Dr. W. Schopman, Rotterdam/

Rotterdam, Holland) was raised in the rabbit against androstenedione-11 $\alpha$ -hemisuccinyl-BSA and used at an initial dilution of 1:10,000. The antiserum showed negligible cross reactions with steroids likely to be encountered in plasma except 11 $\alpha$ -hydroxyandrostenedione (36%), andrenosterone (43%) and testosterone (0.3%) (Baird et al. 1974).

The testosterone antiserum (E01, a gift from Dr. S.A. Tillson, Alza Corporation, Palo Alto, U.S.A.) was raised in the goat against testosterone-3-(O-carboxymethyl)oxime-BSA and used at an initial dilution of 1:6000. The antiserum showed a cross reaction of 25% with 5 $\alpha$ -dihydrotestosterone, 0.2% with oestradiol-17 $\beta$  and 0.08% with androstenedione (Corker & Davidson, 1976).

### 3.1.5. THE CAPACITY OF JUGULAR VENOUS PLASMA AND OVARIAN FOLLICULAR FLUID TO BIND EXOGENOUS TRITIATED STEROIDS.

#### (a) The titre of steroid antibodies.

The binding of four steroids (oestradiol-17 $\beta$ , oestrone, androstenedione and testosterone) by jugular venous plasma from ewes actively immunized with steroid-protein conjugates was determined by incubation of the plasma with tritiated steroid at 4°C and separation of the free and bound steroids by charcoal absorption according to the method of Scaramuzzi et al (1975).

<sup>3</sup>H-1,2- testosterone was obtained from New England Nuclear. <sup>3</sup>H-2,4,6,7-oestrone, <sup>3</sup>H-1,2,6,7-androstenedione and <sup>3</sup>H-6,7-oestradiol-17 $\beta$  were all obtained from the Radiochemical Centre, Amersham. All steroids were stored undiluted at 4°C until use.

A range of dilutions of plasma were prepared in PGB. Duplicate aliquots (0.1ml) were incubated overnight at 4°C with 0.1ml of the tritiated steroid (diluted to give 15-20pg/0.1ml in PGB). The free and bound steroids were separated by the addition of /

of 1ml of a charcoal suspension (250mg charcoal and 25mg dextran/100ml of PGB). After standing for 20 minutes, the tubes were centrifuged and the supernatant decanted and the radioactivity measured. The titre of steroid antibodies, defined as the initial dilution of plasma which bound 50% of the radioactive tracer, was determined from a graph of the percentage of radioactivity bound (Y-axis) against the dilution of plasma (log scale) (X-axis).

The binding of tritiated steroids by a 1:200 dilution of follicular fluid from the ovaries of ewes immunized against oestradiol, oestrone and testosterone was determined as described above.

The removal of free steroid by charcoal absorption disrupted the equilibrium of the system and also removed steroid from the more labile steroid-albumin complexes. In the context of the present studies, the steroid binding determined by the present method represented the high affinity binding of antibodies and serum binding proteins.

(b) The binding of exogenous steroids by jugular venous plasma after equilibrium dialysis at 37°C.

The binding of tritiated steroids by jugular venous plasma was determined by the equilibrium dialysis method of Forest, Rivarola and Nigeon (1968).

Seven inch segments of cellophane dialyzing tubing (Visking Tubing, Scientific Instrument Centre Ltd., London, 7/32" diameter) were immersed in distilled water 30 minutes before use. 1ml of plasma (diluted 1:5 with physiological saline) was placed inside the case and the ends secured with double knots. The bags were placed in 50ml flasks containing 10ml of 0.9% NaCl and 0.1ml of the tritiated/

tritiated steroid (15-25pg). The flask was covered with parafilm and placed in a gyratory shaker (140 strokes/minute) for 20 hours at 37°C.

Duplicate aliquots of the inside solution (0.25ml) and the outside solution (1ml) were placed in plastic counting vials. After the addition of 0.75ml of saline to the vials containing the inside solution, 10ml of the scintillation fluid was added to all vials and the radioactivity measured.

The percentage of steroid bound was calculated by the formula of Slaunwhite (Forest et al. 1968):

$$\% \text{steroid bound} = \left( 1 - \frac{\text{cpm outside} \times \text{volume inside}}{\text{cpm inside} \times \text{volume outside}} \right) \times 100$$

In contrast to the determination of anti-steroid titres described above, the measurement of steroid binding by equilibrium dialysis at 37°C attempted to simulate the binding of steroids by plasma under physiological conditions. Therefore, the percentage of steroid bound was a result of both high affinity antibody binding and the relatively low affinity steroid-albumin interactions (Sandberg, Slaunwhite & Antoniadis, 1957; Wu, Motohashi, Abdel-Rahman, Flickinger & Mikhail, 1976).

### 3.2. EXPERIMENTAL PROCEDURES

#### 3.2.1. ANIMALS.

Twenty-five Welsh mountain ewes were used in the experimental studies described in the present investigations. The animals were kept under field conditions at the Animal Breeding Research Organization Dryden Field Station, Roslin, Midlothian. Supplementary feeding with hay and grain was given during the winter months.

The animals were divided into 5 groups of equal size. Each group was immunized with one of the following compounds:

(1)/



- (1) BSA (the control group).
- (2) 17 $\beta$ -oestradiol-6-(O-carboxymethyl)oxime-BSA (E<sub>2</sub>-6-BSA).
- (3) Oestrone-6-(O-carboxymethyl)oxime-BSA (E<sub>1</sub>-6-BSA).
- (4) Androstenedione-11 $\alpha$ -hemisuccinyl-BSA (A-11-BSA).
- (5) Testosterone-3-(O-carboxymethyl)oxime-BSA (T-3-BSA).

Androstenedione-11 $\alpha$ -hemisuccinyl-BSA was initially prepared by Dr. G. Woods, Organon Laboratories, Newhouse, Lanarkshire, and Dr. F. Rutherford, M.R.C. Radioimmunoassay Team, Edinburgh. On exhaustion of its supply, a second preparation of A-11-BSA together with the other steroid-protein conjugates was generously supplied by Dr. P.D.G. Dean, Department of Biochemistry, University of Liverpool. All conjugates were used without further purification.

The animals were immunized by the procedure of Scaramuzzi et al. (1975).

The antigen was dissolved in 2ml of physiological saline and emulsified in an equal volume of Freund's complete adjuvant (Difco Laboratories). The emulsion was administered by subcutaneous injection at 8 sites. 0.5ml of Pertussis vaccine was given by intramuscular injection and served as an additional adjuvant. Booster immunizations were given at intervals after the primary immunization. The individual dose of antigen and the time of booster immunizations are outlined in the experiments described in the following chapters. Although abscesses sometimes formed at the site of injection, the animals remained healthy and deaths which occurred were unrelated to the immunization procedure.

### 3.2.2. BLOOD SAMPLING.

The animals were brought inside and housed individually in metabolism cages which allowed the animals to sit or stand but prevented them from turning around. Wool was removed from the neck region/

region to expose the jugular vein. The jugular vein was occluded by pressure and an indwelling cannula (Branula, Armour Pharmaceuticals) inserted into the vein and held in place by a suture. Polythene manometer tubing (Portex Ltd.), flushed with physiological saline (Travenol Laboratories Ltd.) containing Heparin (Weddel Pharmaceuticals) (25,000 I.U./500ml), was attached to the cannula and tied to the wool at the rear of the head and the middle of the back. The end of the cannula was suspended at the back of the cage and a 3-way tap (Pharmaseal) attached.

This arrangement enabled the animals to move without severing the tubing. Meal pellets and water were supplied throughout the sampling period. Initially, to avoid blood clots forming in the tip of the cannula, the animals were heparinized with 25,000 I.U. of heparin one hour before the start of blood sampling. This step was found to be unnecessary and subsequent experiments were performed without this procedure.

Blood samples were obtained by the following sequence:

- (1) Heparinized saline present in the cannula was drawn into a 5ml syringe until blood entered the syringe.
- (2) The required volume of blood was drawn into another syringe.
- (3) Heparinized saline was given to replace the volume of blood removed.

This procedure ensured the cannula was filled with heparinized saline and prevented the formation of blood clots between successive blood samples. The whole procedure took approximately 1 minute. Postural obstruction of the cannula due to the position of the animal's head causing the tip of the cannula to be pressed against the wall of the vein was encountered. This was easily overcome by altering the position of the animal's head before removal of the/

the blood sample.

Experiments during the breeding season were performed on several days during the oestrous cycle. At the end of each sampling period, the manometer tubing was removed and a 3-way tap attached to the cannula. The animal was released from the metabolism cage and kept in a pen. The cannula was flushed daily with heparinized saline. Using this procedure, the cannula remained patent between successive sampling periods.

The blood was placed in heparinized tubes and centrifuged at 8°C. The plasma was removed and stored at -20°C until assayed.

### 3.2.3. THE DETECTION OF OESTRUS.

The experiments performed during the breeding season necessitated the detection of oestrus. A vasectomized ram was introduced twice daily into a pen containing the ewes from two days before until two days after the expected oestrus. The behaviour of the ram and the ewes was observed for 20 minutes. A ewe which allowed the ram to mount was defined as being on heat and the time designated as day 0 of the oestrous cycle. The oestrous ewe was removed from the pen before the completion of service to prevent ejaculation and the loss of sexual interest by the ram.

Oestrus was also determined by allowing a vasectomized ram with a mating harness and crayon (Radford, Watson & Wood, 1960) to run with the ewes. The presence of coloured marks on the rump of the ewe indicated an attempted mating. The ewes were examined twice daily for service marks, the time of the first marking being designated as the onset of oestrus.

### 3.2.4. EXPERIMENTAL DESIGN.

Each group of animals was subjected to three experiments. A detailed account of the experimental protocols is described in the following/

following chapters. A brief outline of the experiment, however, is described below.

(i) The concentration of gonadotrophins during anoestrus.

Blood samples were taken at 15-minutes intervals for 12 hours. After an intramuscular injection of 25 or 50 $\mu$ g of oestradiol benzoate (Organon Laboratories Ltd.) (excluding the oestradiol-immunized group), sampling continued at hourly or 2-hourly intervals for 36 or 48 hours respectively. The concentration of LH was determined in every sample and FSH measured in alternate samples.

(ii) The concentration of gonadotrophins during the oestrous cycle.

Blood samples were taken at 15-minute intervals for a 6-hour period at each of 4 days during the oestrous cycle. Plasma LH was measured in every sample and FSH determined in alternate samples.

(iii) Ovarian morphology and steroid secretion.

The ewes were laparotomized during the luteal phase of the oestrous cycle. Samples of jugular venous and ovarian venous blood were removed for steroid analysis. The size of the follicles were measured and the ovaries removed, weighed and fixed in Bouin's fixative for subsequent histology.

3.2.5. STATISTICAL ANALYSIS.

(i) LH concentrations.

The pulsatile nature of LH release was evident in the results obtained in the present study. The occurrence of spontaneous peaks of LH secretion resulted in a skewed distribution of the LH concentrations measured at 15-minute intervals. As a result, the arithmetic mean of this distribution was unsuitable for statistical analysis. To avoid this problem and to highlight the characteristics of LH secretion, several definitions have been introduced.

The/

The mean basal level of LH was defined as the mean of the five lowest concentrations of LH measured during the sampling period.

A peak of LH was defined by the occurrence of at least 2 consecutive concentrations of LH greater than, or equal to 2 standard deviations of the mean basal level. The peak height was obtained after the subtraction of the mean basal level from the maximum concentration of LH occurring during the peak.

The positive feedback action of oestradiol benzoate was defined as the occurrence of at least 5 concentrations of LH greater than 2 standard deviations of the mean basal level of LH occurring after the administration of oestradiol benzoate. The onset of positive feedback, therefore, was determined by the time after oestradiol administration at which the concentration of LH reached 2 standard deviations above the mean basal level.

The negative feedback action of oestradiol was determined by the percentage depression of the mean basal level after oestradiol administration compared to the pre-injection level:

$$\frac{\text{Post-oestradiol mean basal level}}{\text{Pre-oestradiol mean basal level}} \times 100$$

(ii) FSH concentrations.

The concentrations of FSH formed an essentially homogeneous distribution and the arithmetic mean was determined.

Positive and negative feedback responses to oestradiol benzoate were defined essentially as described above, using, however, the mean levels instead of the mean basal level.

Using these parameters, significance was determined by the Student's 't'-test. In most cases, however, the range of response to the immunization procedure between animals of the same group was too/

too great to enable analysis by statistical tests. In such cases, the results from individual animals were compared with the pooled mean from the control group. Results lying outside 2 standard deviations above or below the control mean were regarded as significantly different from the control group.

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CHAPTER 4

THE EFFECTS OF ACTIVE IMMUNIZATION AGAINST  
ANDROSTENEDIONE - 11 $\alpha$  - HEMISUCCINYL - BOVINE SERUM  
ALBUMIN ON THE CONCENTRATION OF GONADOTROPHINS AND  
OVARIAN STEROIDS IN THE EWE.



Androstenedione, a weak androgen, is quantitatively the major C<sub>19</sub> steroid secreted by the ovary in sheep at oestrus (Baird et al. 1968b; Scaramuzzi, Baird, Land & Wheeler, 1974) where it is thought to be the major precursor of oestradiol-17 $\beta$  (oestradiol) (Rado, Baird & McCracken, 1970). Androstenedione is also secreted by the adrenal gland in sheep (Baird, Horton, Longcope & Tait, 1968a; Baird et al. 1973).

The ovarian secretion of androstenedione is closely related to the secretion of oestradiol during the oestrous cycle (Baird et al. 1976a). Furthermore, a simultaneous increase in the secretion rate of both androstenedione and oestradiol occurs in response to exogenous gonadotrophins (Baird et al. 1968b; McCracken et al. 1969) and after spontaneous discharges of LH during the luteal phase of the cycle (Baird, Swanston & Scaramuzzi, 1976b).

The role of circulating androstenedione has not been clearly defined. Androstenedione may act as a prehormone through its extraglandular conversion to oestrone (Baird et al. 1968a). The involvement of androstenedione in the maintenance of sexual receptivity in the female rhesus monkey has been demonstrated (Everitt & Herbert, 1971). A similar role in subprimate species, however, has not been described. A brief communication by Skinner, Mann and Rowson (1968) suggested that androstenedione may be involved in the process of puberty in male calves.

The present study was undertaken to investigate the effects of the neutralization of androstenedione by active immunization on the patterns of gonadotrophin release and on the morphology of the/

the ovary and ovarian steroid secretion in the ewe.

Five Welsh mountain ewes were actively immunized in October, 1972, with 2.1mg of androstenedione-11 $\alpha$ -hemisuccinyl-bovine serum albumin (A-11-BSA) as described previously. Another five ewes were immunized with 2.1mg of bovine serum albumin (BSA) and served as controls. Booster immunizations (1-2mg of antigen) were given to all animals at 155, 620, 785 and 981 days after the primary immunization.

Regular oestrous cycles occurred in the androstenedione-immunized ewes during the breeding season with a mean ( $\pm$  S.E.) cycle length of  $17.8 \pm 0.5$  days ( $n=30$ ). The cycle length was not significantly different from the length of the cycle in the control animals ( $17.1 \pm 0.3$  days;  $M \pm$  S.E.;  $n=43$ ).

#### 4.2 THE EFFECTS OF ACTIVE IMMUNIZATION AGAINST A-11-BSA ON THE PLASMA GONADOTROPHIN CONCENTRATIONS IN EWES DURING ANOESTRUS.

##### 4.2.1. PROCEDURE.

In June, 1974, 14 days after the second booster immunization, serial blood samples were obtained from the ten androstenedione-immunized and control ewes. To examine the patterns of gonadotrophin release in the absence of all ovarian steroids, a group of 5 Welsh mountain ewes that had been ovariectomized and hysterectomized 9 months previously were included in the experiment.

Blood samples (3ml) were obtained from indwelling jugular vein cannulae at 15-minute intervals for 12 hours (1300-0100h). All ewes then received 25 $\mu$ g of oestradiol benzoate (OB) in 1ml of/

of arachis oil (0100h). Sampling continued at hourly intervals for 36 hours.

The concentration of LH was measured in all samples taken from each animal throughout the experiment. The level of FSH was determined in alternate samples taken from each of 3 animals from each group.

The titres of steroid antibodies were determined in the first sample taken from each of the androstenedione-immunized and control ewes.

The binding of steroids after equilibrium dialysis was measured in plasma from the androstenedione-immunized and control animals after pooling the samples taken from each group.

#### 4.2.2. RESULTS.

##### (1) The steroid binding by plasma from the control and androstenedione-immunized ewes.

The steroid antibody titres determined by charcoal absorption in ewes actively immunized against A-11-BSA are shown in Table 4.1. The titres of androstenedione and testosterone antibodies, ranging from 1:600 to 1:5000 and 1:50 to 1:210 respectively, were significantly higher than the binding of these steroids in the control group (Table 4.1).

Equilibrium dialysis at 37°C enabled the measurement of plasma steroid binding under simulated physiological conditions. The percentage of steroid bound by a 1/5 dilution of plasma from the control and androstenedione-immunized ewes are shown in Table 4.2. The percentage of bound androstenedione, testosterone and oestrone was significantly higher in the androstenedione-immunized animals (Table 4.2). The binding of oestradiol, however/

TABLE 4.1.  
THE TITRE<sup>a</sup> OF STEROID ANTIBODIES IN EWES IMMUNIZED AGAINST A-11-BSA

CONTROL	<sup>3</sup> H-Androstenedione	<sup>3</sup> H-Oestradiol	<sup>3</sup> H-Oestrone	<sup>3</sup> H-Testosterone
	N.S.	N.S.	N.S.	1:1
ANDROSTENEDIONE- IMMUNIZED				
7R-206	1:5000	1:1	1:2	1:130
7R-176	1:4400	N.S.	1:1	1: 90
7R-374	1:1700	1:1	1:1	1: 60
7R-171	1:650	N.S.	1:1	1: 50
6R-152	1:600	N.S.	1:1	1:270

<sup>a</sup>Titre = The initial dilution of plasma which bound 50% of 15-20pg of tritiated steroid.

N.S. = <50% binding of the tritiated steroid at a dilution of 1:1.

TABLE 4.2.  
THE PERCENTAGE (N.E.S.E.) OF 15-25pg OF TRITIATED STEROID BOUND BY A 1/5 DILUTION OF PLASMA  
FROM THE CONTROL AND ANDROSTENEDIONE-IMMUNIZED EWES DETERMINED BY EQUILIBRIUM DIALYSIS

	$^3\text{H}$ -ANDROSTENEDIONE	$^3\text{H}$ -OESTRADIOL	$^3\text{H}$ -OESTRONE	$^3\text{H}$ -TESTOSTERONE
CONTROL	57.6+1.3 (n=5)	84.9+1.9 (n=5)	71.4+2.3 (n=5)	75.4+2.1 (n=5)
ANDROSTENEDIONE- IMMUNIZED	99.4+0.1* (n=5)	86.4+2.3 (n=5)	95.6+1.7* (n=5)	99.0+0.3* (n=5)

\*  $p < 0.001$ . Immunized vs. Control (Student's t-test).

however, was essentially the same in both groups.

A comparison of the percentage of steroid bound by a 1/5 dilution of plasma measured by the two methods, charcoal absorption and equilibrium dialysis is shown in figure 4.1. The binding of all steroids in the control group and oestradiol and oestrone in the androstenedione-immunized group was very much higher when determined by equilibrium dialysis (fig. 4.1). The binding of androstenedione and testosterone by plasma from the androstenedione-immunized ewes, however, was similar when measured by either method (fig. 4.1).

(2) The plasma concentrations of LH and FSH in control ewes during anoestrus.

The concentrations of LH and FSH in two ewes, representative of the control group are shown in figure 4.2.

During the 12-hour period before OB administration, the mean basal level of LH was low in all control animals (range:  $<0.12$ - $0.34$  ng/ml). A spontaneous discharge of LH, characterized by a rapid increase followed by an exponential decline in the concentration of LH (e.g. 8R-542, fig. 4.2), occurred in 4 out of the 5 control animals. The peak of LH occurred at random throughout the 12-hour period with a duration of 1-2 hours and peak heights ranging from  $5.70$ - $12.50$  ng/ml. The fifth ewe (8R-621, fig. 4.2) failed to show any spontaneous peak in the concentration of LH which remained beneath the sensitivity of the assay ( $<0.12$  ng/ml) throughout the 12-hour period.

Following the injection of  $25\mu\text{g}$  of OB, the concentration of LH remained low until the onset of positive feedback which occurred/

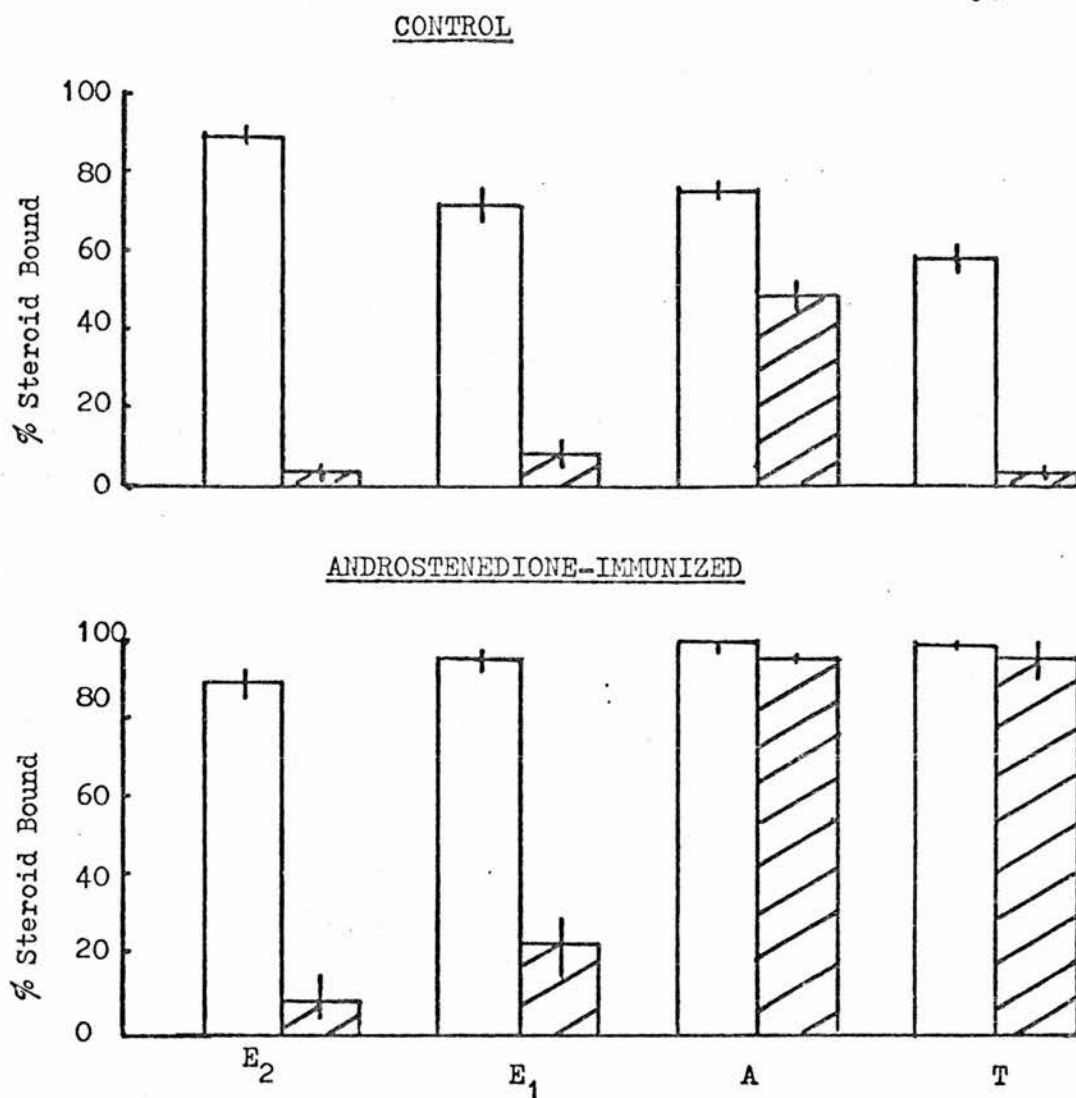


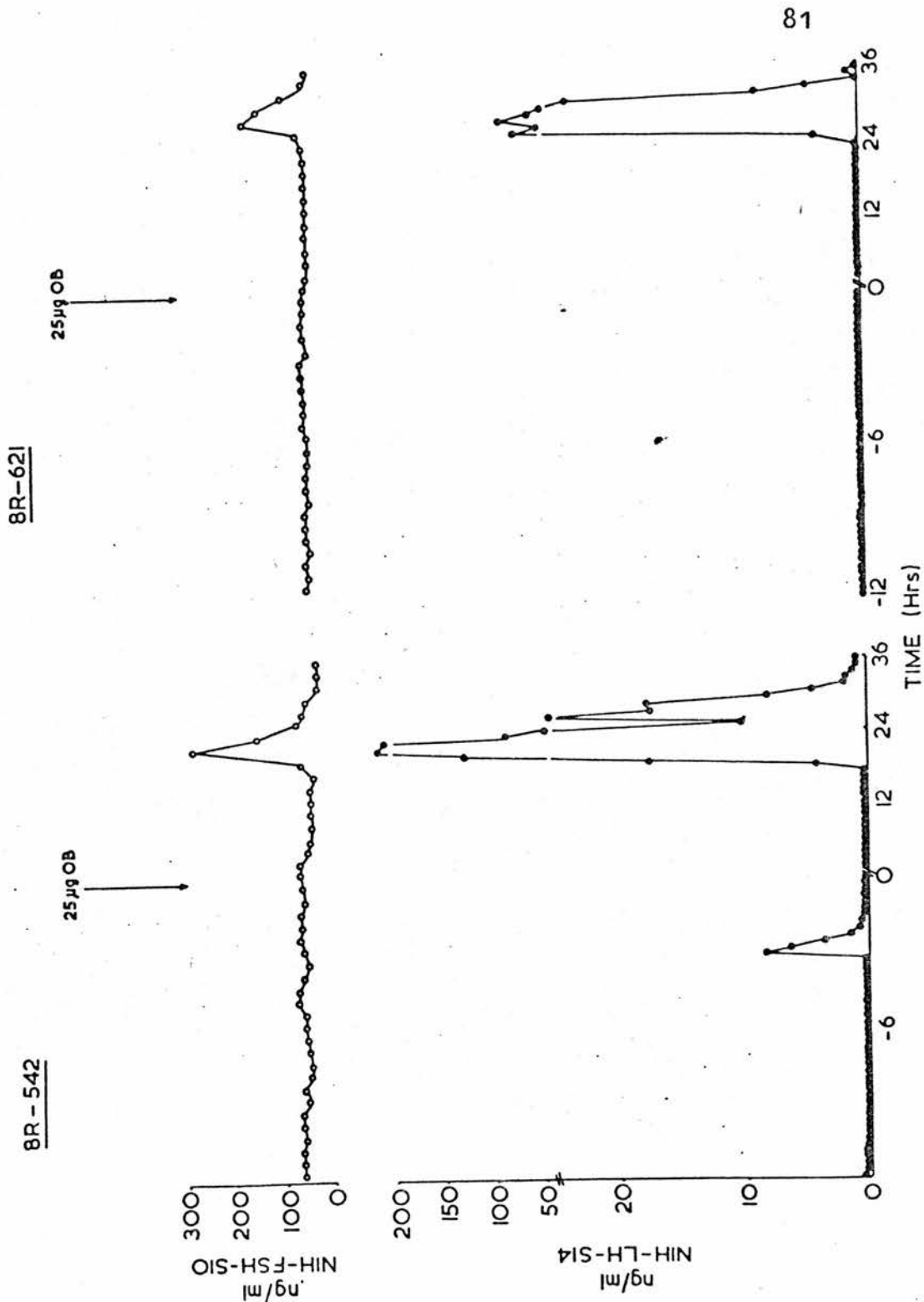
Figure 4.1. The steroid binding determined by equilibrium dialysis and charcoal absorption by a 1/5 dilution of plasma from control and androstenedione-immunized ewes.

The open bar represents steroid binding after equilibrium dialysis.  
The hatched bar represents steroid binding after charcoal absorption.

E<sub>2</sub> = oestradiol; E<sub>1</sub> = oestrone; A = androstenedione;  
T = testosterone.



**Figure 4.2.**  
The plasma concentrations  
of LH and FSH in two control  
ewes during anoestrus.  
 Samples were taken at  
 15-minute intervals for  
 12 hours before an  
 injection of 25 $\mu$ g of  
 oestradiol benzoate.  
 Sampling then continued  
 at hourly intervals for  
 36 hours.  
 LH was assayed in every  
 sample.  
 FSH was measured in alternate  
 samples.



occurred in all control animals  $19.4 \pm 1.7$  hours ( $M \pm S.E.$ ;  $n=5$ ) after OB treatment. The concentration of LH during positive feedback reached a maximum of  $185.6 \pm 45.3$  ng/ml ( $M \pm S.E.$ ;  $n=5$ ) and remained significantly higher than the post-OB baseline level for at least 10 hours.

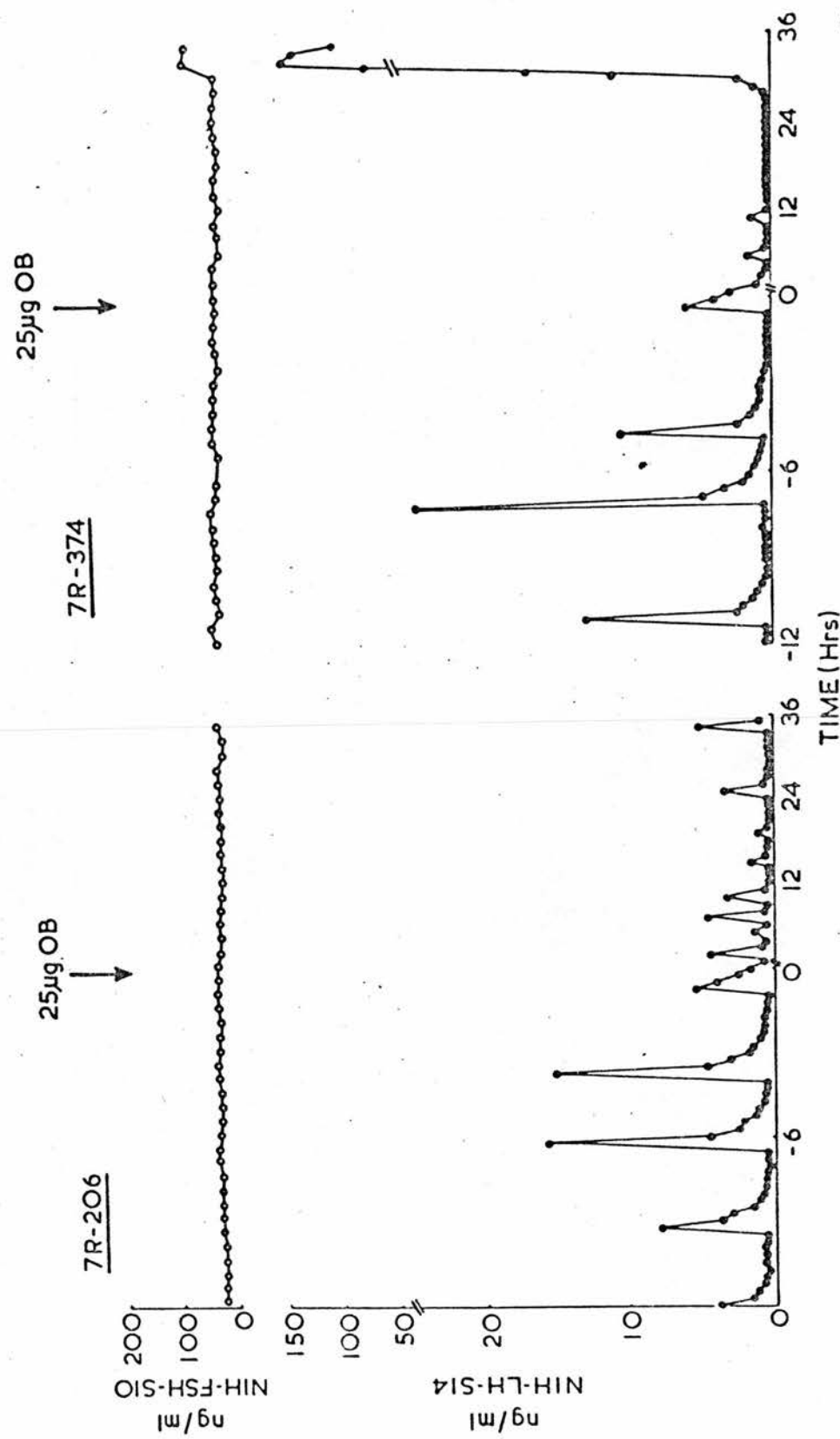
No distinct peaks in the concentration of FSH were apparent during the 12-hour period before OB administration in the 3 ewes in which FSH was assayed (e.g. fig. 4.2). The mean concentration of FSH ranged from 39.2 ng/ml to 60.6 ng/ml.

The positive feedback response of FSH to oestradiol occurred in the 3 control ewes at a time coincident with the LH surge ( $19.3 \pm 9.7$  hours;  $M \pm S.E.$ ;  $n=3$ ) (fig. 4.2). The level of FSH following OB administration (before the onset of positive feedback) was reduced to 80% of the pre-injection level suggesting that the negative feedback response of FSH to oestradiol was operable.

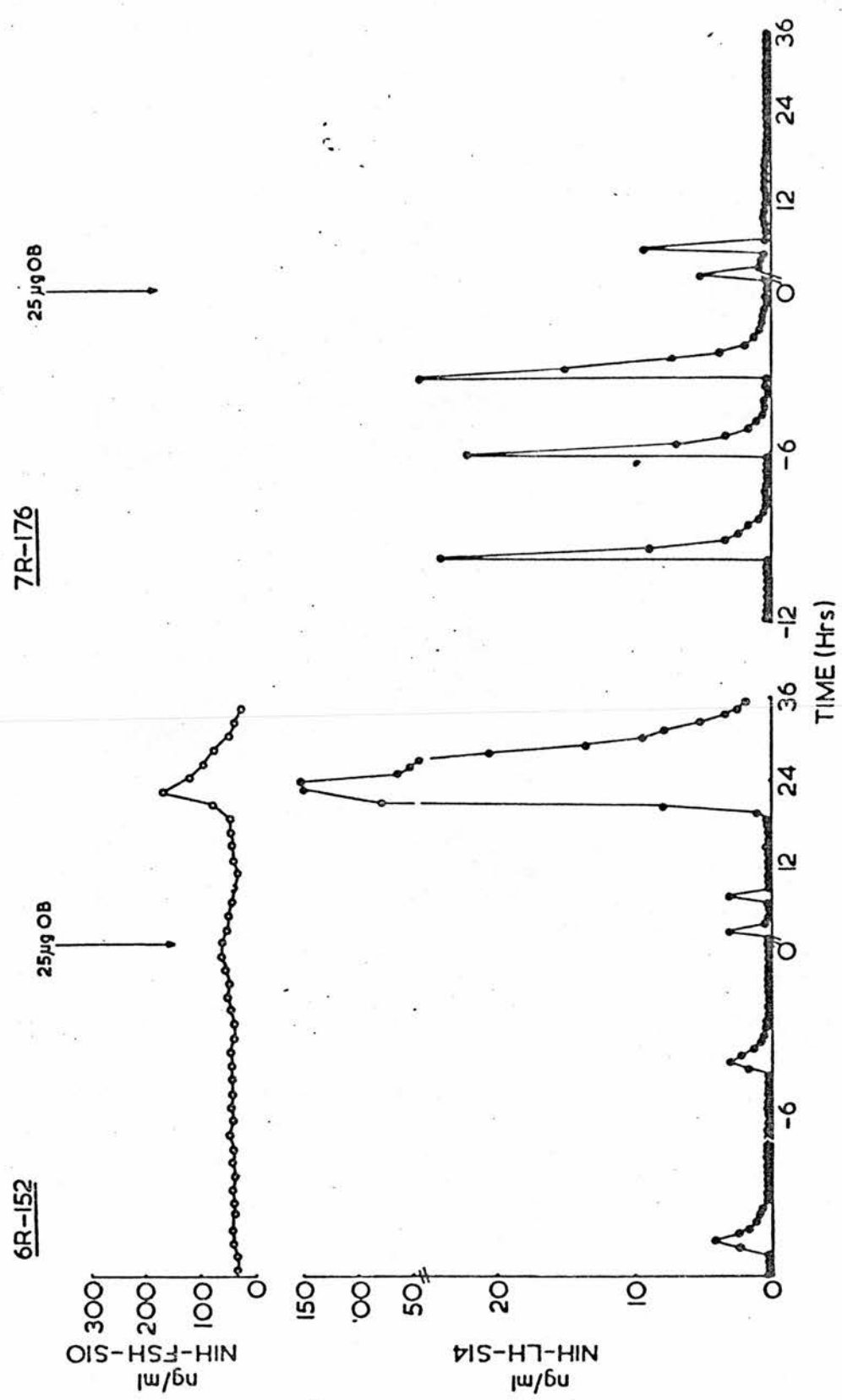
(3) The plasma concentrations of LH and FSH during anoestrus in ewes actively immunized against A-11-BSA.

The concentrations of LH in four of the androstenedione-immunized ewes are shown in figures 4.3 and 4.4. Although the mean basal level of LH ( $<0.12-0.24$  ng/ml) was similar to that found in the control group, the number of spontaneous discharges of LH occurring during the 12-hour period before OB administration was increased (figs. 4.3 and 4.4). A positive correlation between the number of LH peaks and the titre of androstenedione antibodies was apparent (fig. 4.5b). The duration of the LH peaks (1-2 hours) and the mean peak height ( $10.91 \pm 2.10$  ng/ml;  $M \pm S.E.$ ;  $n=15$ ) were not significantly different/

**Figure 4.3.**  
The plasma levels  
of LH and FSH in two  
androstenedione-  
immunized ewes during  
anestrus.  
 Samples were taken at  
 15-minute intervals  
 before an injection of  
 25 $\mu$ g of oestradiol  
 benzoate. Sampling  
 then continued at  
 hourly intervals for  
 36 hours.



**Figure 4.4.**  
The plasma levels of  
LH and FSH in  
androstenedione-  
immunized ewes  
during anoestrus.  
 Samples were taken at  
 15-minute intervals for  
 12 hours before an  
 injection of 25 $\mu$ g of  
 oestradiol benzoate.  
 Sampling then  
 continued at hourly  
 intervals for 36 hours.  
 LH was assayed in all  
 samples.  
 FSH was measured in  
 alternate samples.



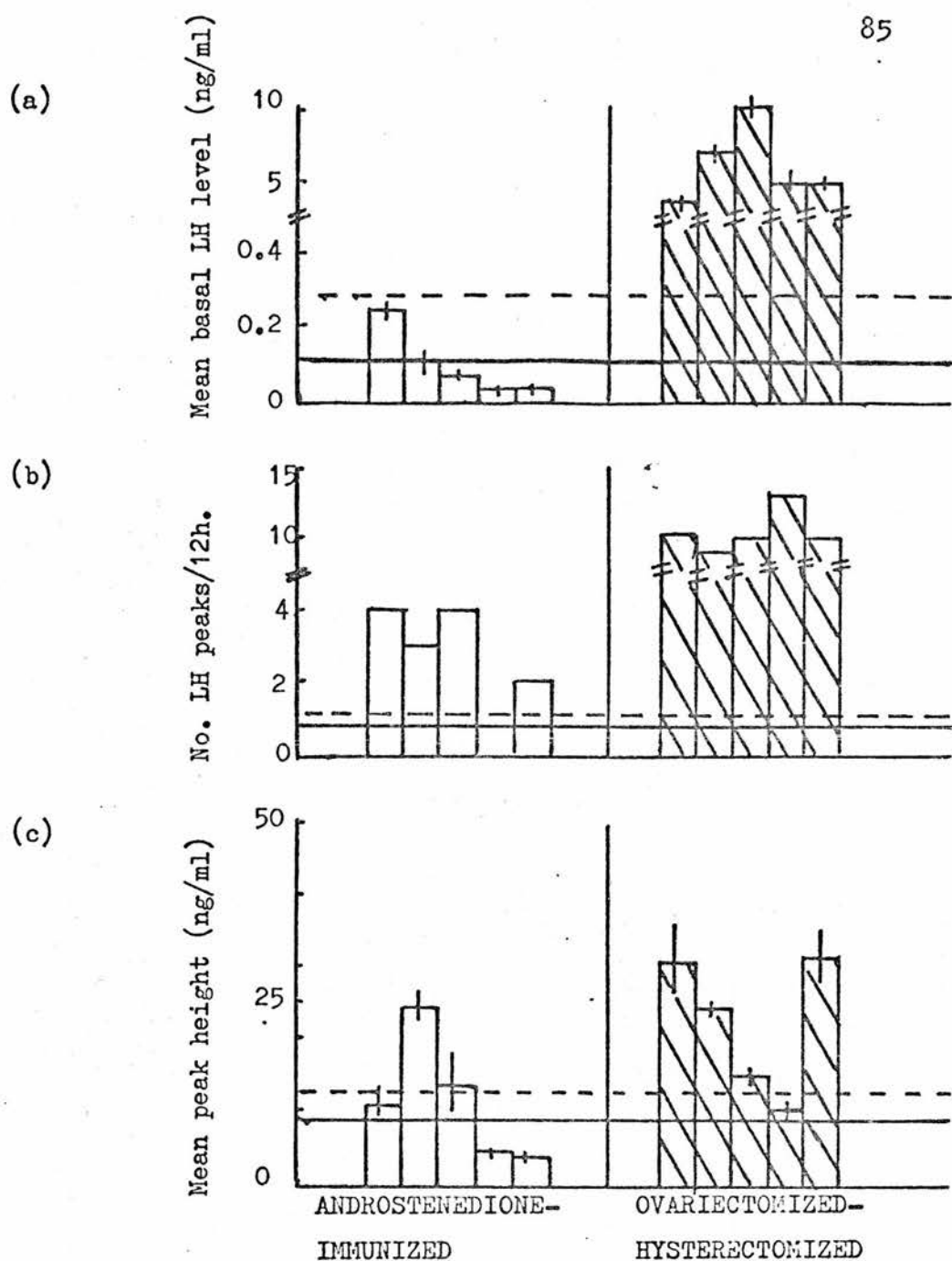


Figure 4.5. The pattern of LH release in androstenedione-immunized and ovariectomized-hysterectomized ewes during anoestrus.

The values from the androstenedione-immunized animals are arranged in order of decreasing androstenedione antibody titre.

The horizontal line represents the mean, and the broken line, the range (mean + 2 S.D.) of the control group.

The vertical line represents the range (mean  $\pm$  S.E.) of individual animals.

different from the control values.

Following the injection of 25 $\mu$ g of OB, the frequency of LH peaks was reduced and positive feedback occurred in 3 out of the 5 animals within 36 hours. Those animals with high titres showed either a delay in the onset of positive feedback (28h) (7R-374, fig. 4.3) or the complete absence of positive feedback within 36 hours of OB (7R-206, fig. 4.3; 7R-176, fig. 4.4). The other two animals, with low androstenedione antibody titres showed a normal response at 18 and 21 hours after OB.

The concentrations of FSH in 3 androstenedione-immunized ewes are shown in figures 4.3 and 4.4. No distinct peaks in the concentration of FSH were apparent before OB administration. The mean concentration of FSH in two animals with high androstenedione antibody titres (7R-206 and 7R-374) was significantly lower than the control value (fig. 4.6). The mean level of FSH in the third animal (6R-152) with a low antibody titre lay within the control range (fig. 4.6).

Following OB administration, the concentration of FSH was reduced by 14-25% compared to the mean pre-injection level. The positive feedback response of FSH to oestradiol was present in 2 of the 3 animals (figs. 4.3 and 4.4). The time of onset of positive feedback was coincident with the LH surge in these animals.

(4) The plasma concentrations of LH and FSH during anoestrus in ovariectomized-hysterectomized ewes.

The concentrations of LH and FSH in 3 ovariectomized-hysterectomized/

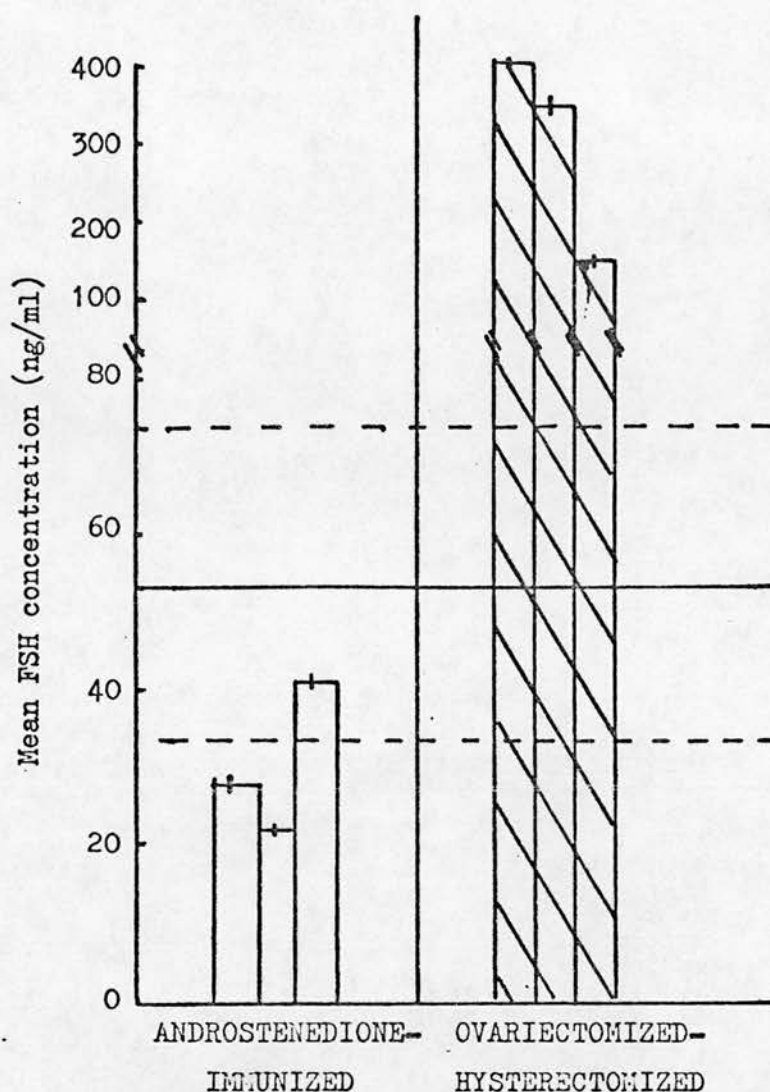


Figure 4.6. The mean plasma concentration of FSH during anoestrus in androstenedione-immunized and ovariectomized-hysterectomized ewes.

The values from the androstenedione-immunized animals are arranged in order of decreasing androstenedione antibody titre.

The horizontal line represents the mean, and the broken line the range (mean  $\pm$  2 S.D.) of the control group.

The vertical line represents the range (mean  $\pm$  S.E.) of individual animals.



hysterectomized ewes during anoestrus are shown in figures 4.7 and 4.8.

All ewes showed a pulsatile pattern of LH release with 9-13 peaks of LH during the 12-hour period before OB. The mean basal level of LH in all ewes was significantly higher than the control value (fig. 4.5a).

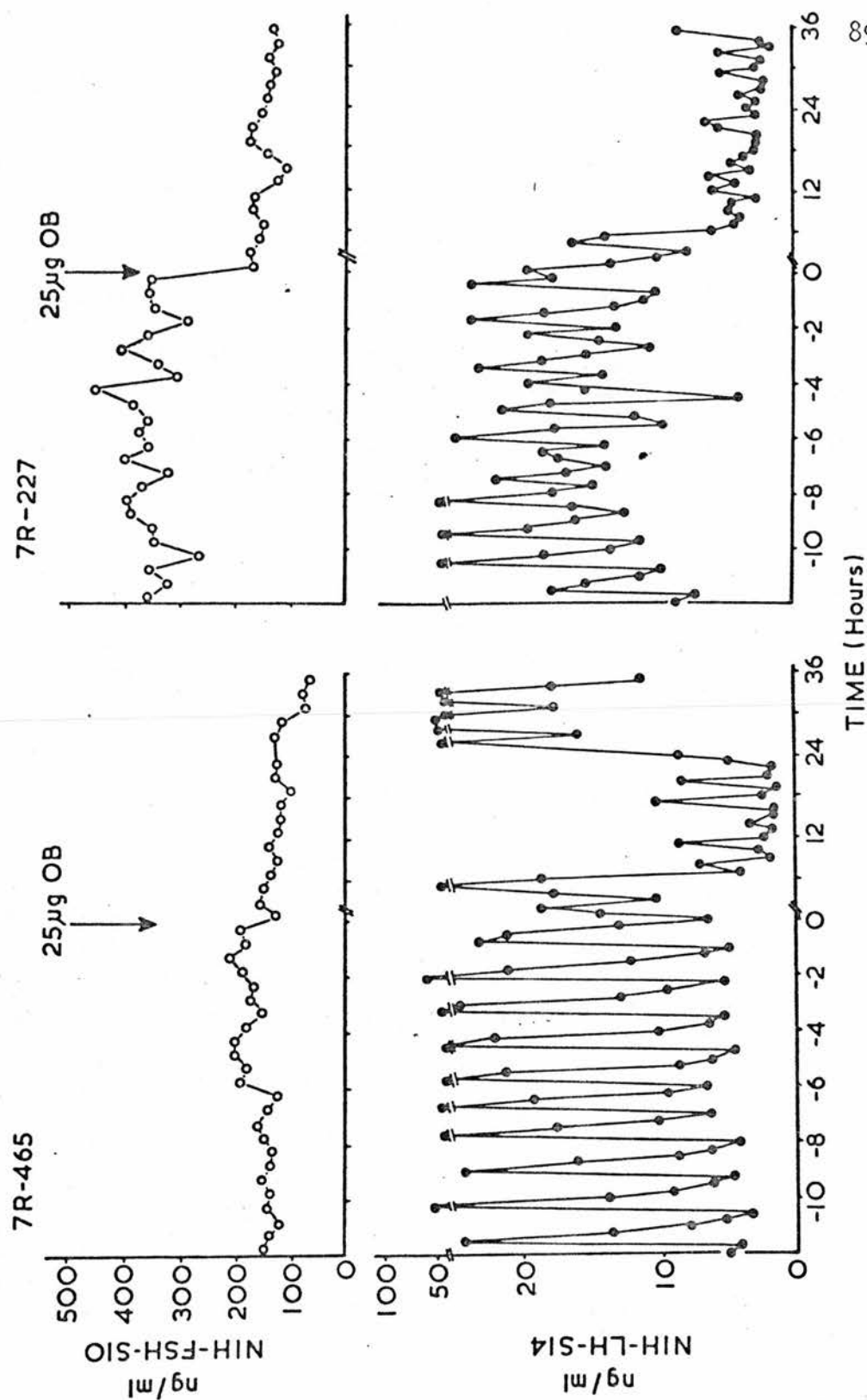
Immediately after OB administration, the episodic release of LH was suppressed and the concentration of LH fell to 71% (range: 66-76%) of the pre-injection value. A positive feedback surge of LH occurred in 4 out of the 5 ewes  $19.5 \pm 1.6$  hours ( $M \pm S.E.$ ;  $n=4$ ) after the injection of OB. The fifth animal (7R-227, fig. 4.7) failed to show positive feedback within 36 hours of OB.

The concentrations of FSH determined in 3 ovariectomized-hysterectomized ewes are shown in figures 4.7 and 4.8. The concentration of FSH fluctuated at random and no correlation between these fluctuations in FSH and the peaks of LH was apparent. The mean concentration of FSH in each of the 3 ovariectomized-hysterectomized ewes was significantly higher than the control value (fig. 4.6.).

After the injection of 25 $\mu$ g of OB, the levels of FSH were reduced (figs. 4.7 and 4.8) indicating that the negative feedback action of oestradiol on FSH secretion was operable. The positive feedback response of FSH to oestradiol, however, was only observed in one animal (6R-125, fig. 4.8). The other two animals failed to show positive feedback even though in one animal (7R-465, fig. 4.7), a clearly defined surge of LH was apparent.

**Figure 4.7.**  
The plasma levels of  
LH and FSH in two  
ovariectomized-  
hysterectomized ewes  
during anoestrus.

Samples were taken at  
 15-minute intervals for  
 12 hours before an  
 injection of 25 $\mu$ g of  
 oestradiol benzoate.  
 Sampling continued at  
 hourly intervals for  
 36 hours.



TIME (Hours)

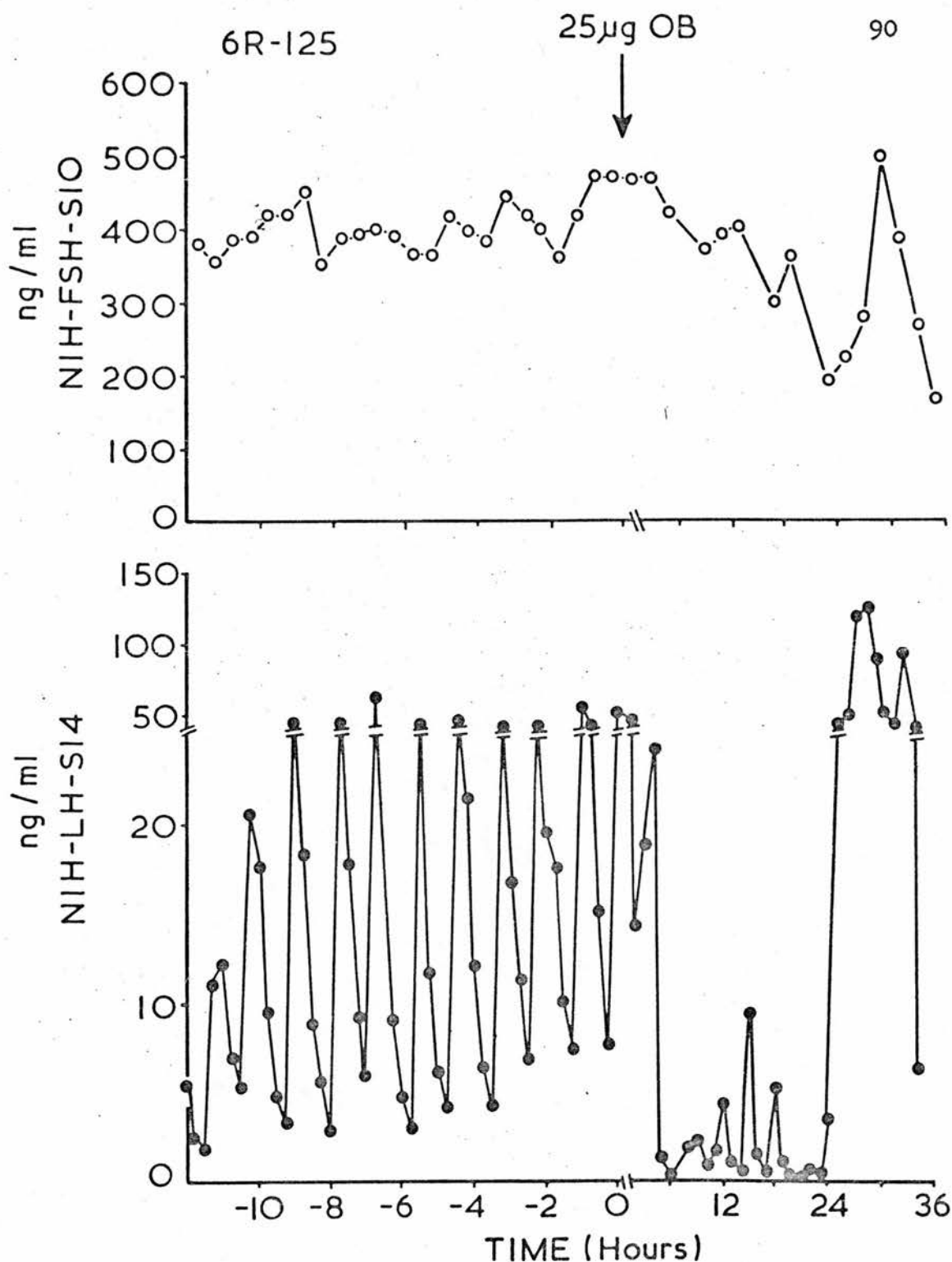


Figure 4.8. The plasma concentrations of LH and FSH during anoestrus in an ovariectomized-hysterectomized ewe.

Samples were taken at 15-minute intervals for 12 hours before an injection of 25µg of oestradiol benzoate. Sampling then continued at hourly intervals for 36 hours. LH was assayed in every sample. FSH was measured in alternate samples.

(1) The plasma steroid binding capacity in ewes actively immunized against A-11-BSA.

The determination of steroid antibody titres by incubation of plasma with radioactive steroid at 4°C and separation of the free and bound steroid by absorption with charcoal has been well established in the field of steroid radioimmunoassay (Ratcliffe, 1974). With this technique, significant titres of androstenedione and testosterone antibodies were demonstrated in the plasma from ewes actively immunized against A-11-BSA. The capacity of the plasma to bind oestrogens, even at low dilutions, was not greatly increased.

The binding of steroids by plasma from the androstenedione-immunized ewes, when determined by equilibrium dialysis, showed a significant increase in the binding not only of androstenedione and testosterone but also of oestrone. The measurement of steroid binding by equilibrium dialysis includes both the high affinity antibody and steroid-binding protein activity and the low affinity steroid-albumin interactions (Sandberg et al. 1957; Wu et al. 1976). Moreover, steroids bound by the latter class of protein could still exert their biological actions (Rosenfield, 1971; Vermeulen, Stoica & Verdonck, 1971). The assessment of steroid binding by equilibrium dialysis for the evaluation of the effectiveness of steroid neutralization in immunological studies would therefore be of limited value.

A comparison between the steroid binding measured by charcoal absorption and equilibrium dialysis showed a marked difference/

difference between the two methods. In control ewes, charcoal absorption abolished virtually all the binding of oestradiol, oestrone and androstenedione seen after equilibrium dialysis. The presence of a significant level of testosterone binding in these animals after charcoal absorption suggested the presence of a specific testosterone-binding protein since charcoal absorption has been used in the assessment of testosterone-binding globulin in other species (Rosenfield, 1971; Wu et al. 1976). In contrast, the binding of androstenedione and testosterone by plasma from the androstenedione-immunized ewes was essentially the same when measured by either method suggesting the presence of high affinity binding of these steroids.

(2) The effects of active immunization against androstenedione on gonadotrophin secretion in the ewe during anoestrus.

The morphology of the ovary in the anoestrous ewe is characterized by the absence of corpora lutea (Grant, 1934; Cole & Miller, 1935; McKenzie & Terrill, 1937) and the presence of small follicles of up to 5mm in diameter (Kammlade, Welch, Nalbandov & Norton, 1952). During the breeding season, follicles of this size are known to secrete small amounts of oestradiol (Moor, 1973) and it is conceivable, therefore, that the same follicles during anoestrus also possess some secretory activity.

The concentration of both androstenedione and oestradiol in utero-ovarian venous plasma was significantly higher than the corresponding concentration in peripheral plasma in two ewes during anoestrus (Martensz, Baird, Scaramuzzi & Van Look, 1976) suggesting/

suggesting that both steroids are actively secreted by the ovary during this period of sexual inactivity.

The fact that ovariectomy during anoestrus is followed by a rapid rise in the plasma concentration of LH (Reeves, et al. 1972) suggests that some secretory product(s) from the ovary is responsible for the low levels of LH found in the anoestrous ewe (Roche, Foster, Karsch, Cook & Dzuik, 1970a; Reeves, Tarnavsky & Chakraborty, 1974<sup>b</sup>). Exogenous administration of oestradiol to anoestrous ewes will induce a positive discharge of LH (Goding et al. 1969; Beck & Reeves, 1973; Nett, Akbar & Niswender, 1974). Furthermore, the presence of physiological levels of oestradiol in ovariectomized ewes with implants of oestradiol is capable of suppressing the concentration of LH to the level seen in intact anoestrous ewes (Karsch et al. 1976).

The question arises as to the role, if any, of androstenedione, a steroid also secreted by the ovary during anoestrus. The peripheral conversion rate of androstenedione to oestrone in men and women is thought to be in the range of 1.5% (Baird et al. 1968a; Naftolin & Ryan, 1975). If a similar rate of conversion occurs in the sheep, the milligram quantities of androstenedione secreted in the course of a day (Baird et al. 1968b) could contribute a significant proportion of the total oestrogens in the ewe. It is conceivable, therefore, that androstenedione, or its metabolites, could modulate the actions of ovarian oestradiol secretion.

The present study examined the effects of the neutralization of biologically active androstenedione on the patterns of LH and FSH release in the anoestrus ewe.

In/

In the absence of all ovarian steroids, LH secretion in the ewe is characterized by the occurrence of frequent pulses of LH (Butler et al. 1972; Diekman & Malven, 1973; Davis & Borger, 1974). The neutralization of biologically active androstenedione increased the frequency of spontaneous LH discharges compared to the intact anoestrous ewe although the frequency was less than that found in ovariectomized-hysterectomized ewes. It is apparent, therefore, that androstenedione may play a role in modulating LH secretion in the anoestrous ewe. Androstenedione, however, is by no means the primary regulatory ovarian steroid in the control of LH secretion.

The administration of exogenous oestradiol resulted in an initial depression of LH secretion followed by a surge of LH in the ovariectomized-hysterectomized ewes. Although the positive surge of LH in response to oestradiol occurred in the control ewes, it is difficult to evaluate the negative feedback action of oestradiol in these animals since the concentration of LH was already very low before oestradiol treatment. These findings confirm the dual role of oestradiol on LH secretion in the ewe.

Although a reduction in the frequency of spontaneous LH discharges following oestradiol administration was apparent in the androstenedione-immunized ewes, suggesting that the negative feedback action of oestradiol was intact, the positive feedback response was delayed or absent in the animals with high titres of androstenedione antibodies. The negative feedback action of oestradiol occurs before the positive feedback action of this steroid (Scaramuzzi et al., 1970a, 1971) suggesting that/



that the threshold for negative feedback is lower than the threshold for positive feedback. Therefore, in the androstenedione-immunized ewes, the negative feedback action of 25 $\mu$ g of OB was evident, but the positive feedback action of this dose of oestradiol was modified in the absence of circulating biologically active androstenedione.

Corroborative evidence in support of the concept of a modulatory role of androstenedione in the positive feedback action of oestradiol was the finding that 5 $\mu$ g of oestradiol benzoate was effective in inducing a surge of LH in anoestrous ewes bearing implants of androstenedione or oestrone, but was ineffective in inducing such a response in control ewes bearing cholesterol implants (P.F.A. Van Look & R.J. Scaramuzzi, Unpublished results).

Although the secretion of LH in the sheep has been studied extensively, the release of FSH has received less attention due to the difficulties in obtaining valid radio-immunoassays for this hormone.

During anoestrus, the plasma concentration of FSH is low compared to the concentration during the breeding season (L'Hermite et al. 1972; Salamonsen et al. 1973). The concentrations of FSH in intact anoestrous and ovariectomized-hysterectomized ewes determined in the present study are within the range of previously reported values (L'Hermite et al. 1972; Salamonsen et al. 1973). The administration of 25 $\mu$ g of OB in the present study produced a surge of FSH coincident with the LH surge in control animals, confirming earlier reports (Jonas, Salamonsen, Burger, Chamley, Cumming, Findlay & Goding, 1973; Nett, et al. 1974; Reeves, Beck & Nett, 1974a). The dose at which/

which oestradiol will cause a reduction in the plasma concentration of FSH has been the subject of conflicting reports. Reeves et al. (1974~~9~~) found that doses of oestradiol less than 100 $\mu$ g were ineffective in causing a reduction in the plasma concentration of FSH although an elevation of FSH could be produced by doses as low as 12.5 $\mu$ g. On the other hand, Jonas et al. (1973) reported that an infusion of oestradiol (10 $\mu$ g/h for 4h) produced a significant reduction in the plasma concentration of FSH. In the present study, a significant reduction in the concentration of FSH after the administration of 25 $\mu$ g of OB to about 80% of the pre-injection level was observed in the control animals.

The unexpected reduction in the plasma concentration of FSH in the androstenedione-immunized ewes to about 50% of the control value is difficult to explain. It is tempting to suggest that androstenedione in some way antagonizes the negative feedback action of oestradiol on FSH release.

It has recently been demonstrated that an "inhibin-like" substance present in bovine follicular fluid will suppress FSH release (without alteration to LH) in ovariectomized rats (De Jong & Sharpe, 1976). An alternative explanation for the depression of FSH in the androstenedione-immunized ewes may lie in an increased production of this follicular "inhibin", perhaps related to the changes in steroid synthesis occurring in these animals, thereby producing the selective suppression of FSH secretion.

The effects of active immunization against androstenedione on the release of FSH in the ewe cannot be explained in terms of established hormonal relationships. Any attempt, at the present/

present time, to explain the reduction of FSH in the androstenedione-immunized animals might well enter the realms of pure hypothesis.

#### 4.3 THE PLASMA CONCENTRATIONS OF LH, FSH AND PROGESTERONE DURING THE OESTROUS CYCLE IN ANDROSTENEDIONE-IMMUNIZED EWES.

##### 4.3.1. PROCEDURE.

The plasma levels of LH, FSH and progesterone during the oestrous cycle were determined in 4 ewes actively immunized against A-11-BSA (one animal, 7R-176, had died since the anoestrous experiment) and the 5 control ewes, 45 days after the third booster immunization.

The occurrence of two consecutive heats was detected by observation of the ram-ewe interactions as described previously.

Indwelling jugular vein cannulae were inserted and remained in situ throughout the experiment. Blood samples (5ml) were taken at 15-minute intervals for 6 hours on each of 4 days during the oestrous cycle (Table 4.3). The sampling days were chosen to enable an examination of the luteal phase of the cycle. One control (8R-542) and one androstenedione-immunized animal (7R-206) were sampled during the two days before oestrus (Table 4.3) thus allowing some measure of the gonadotrophin and progesterone concentrations during the follicular phase of the cycle to be ascertained.

To account for variations in the length of the oestrous cycle, the results were normalized around the day of the second oestrus (day 0).

The/

TABLE 4.3.

THE SAMPLING PROTOCOL DURING THE OESTROUS CYCLE

	Cycle length (days)	Sampling period (days before or after the second oestrus)						
<u>CONTROL</u>								
8R-542	16		-10	-6		-2	+2	
8R-621	17		-12	-8	-4		+1	
8R-602	17		-12	-7	-4		+1	
8R-676	16		-11	-7	-3		+1	
8R-354	17		-12	-8	-4		+1	
<u>ANDROSTENEDIONE- IMMUNIZED.</u>								
6R-152	17		-12	-8	-3		+1	
7R-171	17			-8	-4		+1	+5
7R-206	18	-14	-10		-5	-1		
7R-374	16		-10	-7	-3		+1	

At each sampling period, samples of jugular venous blood were taken at 15-minute intervals for 6 hours.

The plasma concentrations of LH were measured in every sample taken from each animal. The level of FSH was determined in alternate samples from each of 3 ewes from each group.

The concentration of progesterone was measured in the first sample taken from each animal at each sampling period.

Steroid antibody titres, steroid binding after equilibrium dialysis and the total concentrations of oestradiol, oestrone, androstenedione and testosterone were all measured in a 50ml sample taken from each ewe at the end of the final sampling period.

#### 4.3.2. RESULTS.

##### (1) The concentrations of LH, FSH and progesterone during the oestrous cycle in control ewes.

The mean concentrations of progesterone in jugular venous plasma from control ewes during the oestrous cycle are shown in figure 4.9. Low levels of progesterone ( $<0.1-0.4$  ng/ml) were found on the two days after oestrus. The concentration of progesterone then rose during the luteal phase of the cycle to reach maximal levels ( $2.1-2.8$  ng/ml) 3 and 4 days before oestrus. The concentration of progesterone in the ewe sampled two days before oestrus had fallen to  $0.3$  ng/ml.

The concentrations of LH during the oestrous cycle in four control ewes are shown in figures 4.10 and 4.11. Considerable variation in the LH patterns between ewes was apparent. In general, the level of LH was low throughout the luteal phase of the cycle although peaks of LH were observed in some ewes at all/

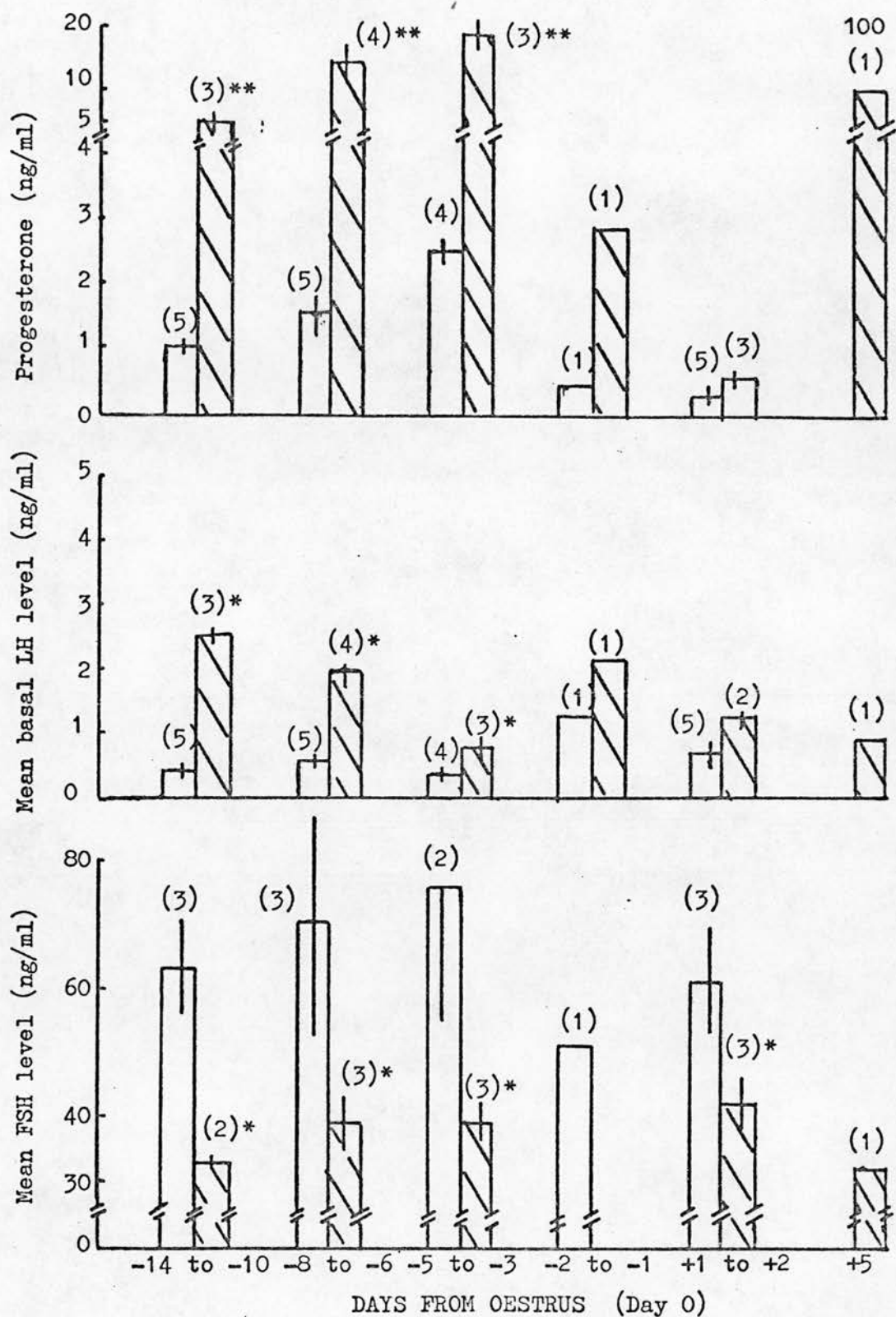


Figure 4.9. The mean concentration of FSH and progesterone and the mean basal level of LH during the oestrus cycle in control and androstenedione-immunized ewes.

The hatched bar represents the androstenedione-immunized value and the open bar, the control value. The vertical line represents the range (mean  $\pm$  S.E.). Significance, Immunized vs. Control: \*\* $p < 0.01$ ; \* $p < 0.05$ . (Student's t-test).



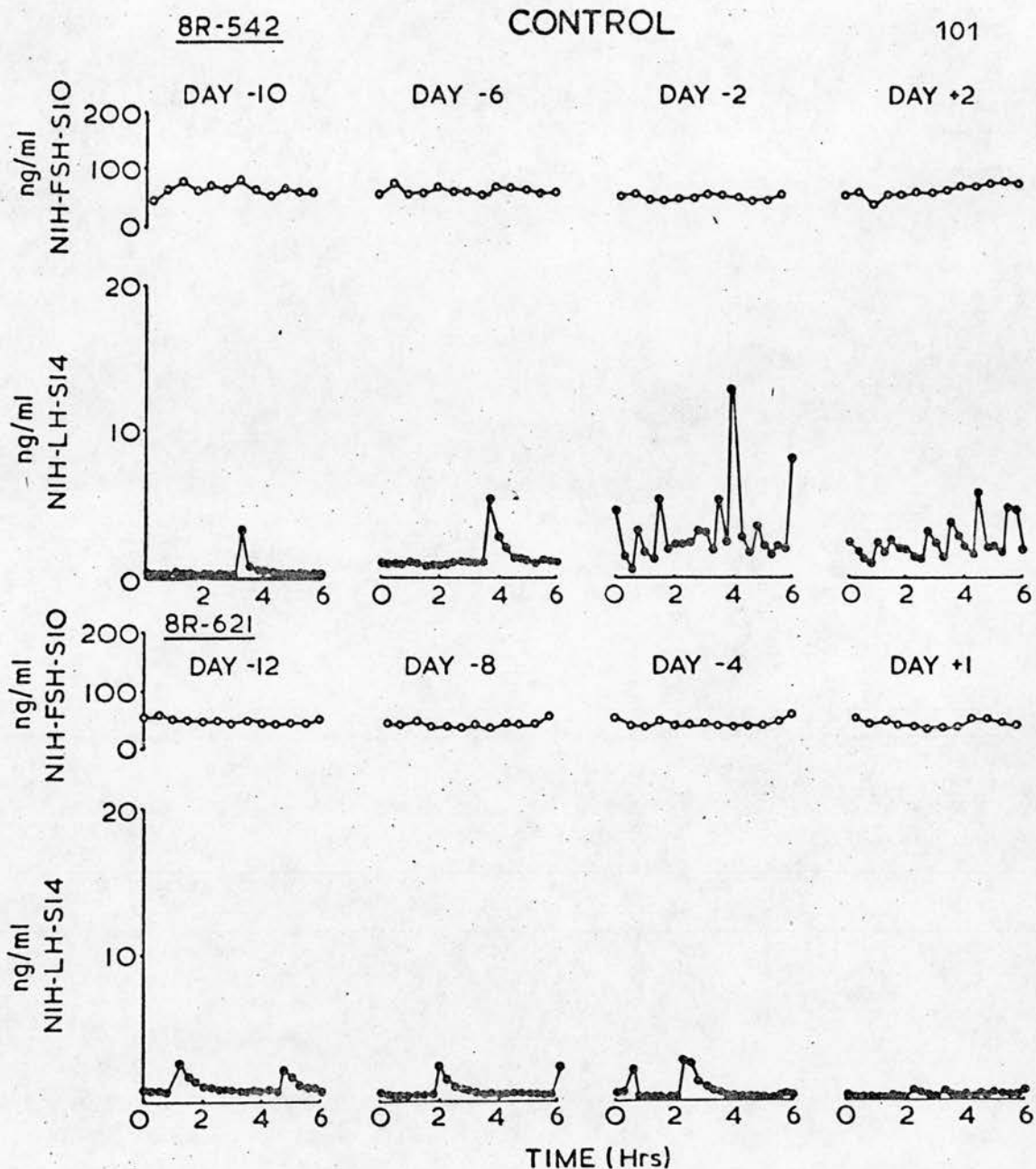


Figure 4.10 The plasma concentrations of LH and FSH during the oestrous cycle in two control ewes.

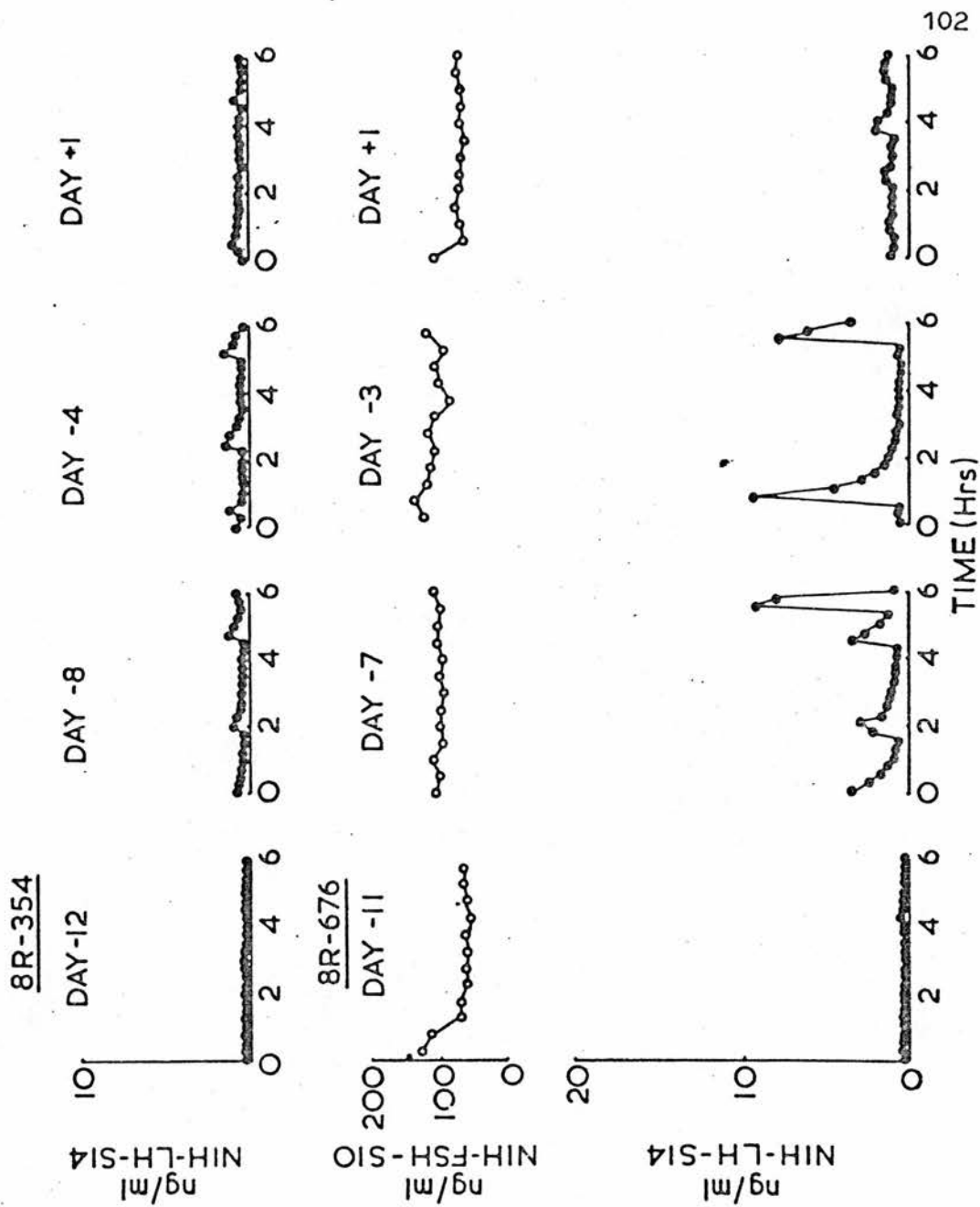
Samples were taken at 15-minute intervals for 6 hours on each of 4 days during the oestrous cycle.

LH was assayed in every sample. FSH was measured in alternate samples.

**Figure 4.11.**

The plasma levels of LH and FSH during the oestrus cycle in control ewes.

Samples were taken at 15-minute intervals for 6 hours on each of 4 days during the oestrous cycle. LH was assayed in every sample. FSH was measured in alternate samples.





all stages. The concentration of LH in the ewe sampled two days before oestrus (7R-542, fig 4.10) was significantly higher than the levels seen during the luteal phase in this animal. The mean basal level of LH in control ewes during the oestrous cycle is shown in figure 4.9. No distinct trend in this parameter of LH release was apparent during the luteal phase of the cycle. The mean basal level of LH in the animal sampled on day -2 was higher than that found at any other period of the cycle examined.

The concentrations of FSH during the oestrous cycle in 3 control ewes are also included in figures 4.10 and 4.11. The concentration of FSH was uniform throughout the cycle within each animal. However, considerable variation in the mean FSH concentration was apparent between animals (range: 46.4-112.0 ng/ml).

(2) The plasma concentrations of LH, FSH and progesterone during the oestrous cycle in ewes actively immunized against A-11-BSA.

The mean concentrations of progesterone during the oestrous cycle in androstenedione-immunized ewes are shown in figure 4.9.

The level of progesterone ( $<0.1-0.5$  ng/ml) in the 3 androstenedione-immunized ewes sampled on day +1 was similar to the control value.

During the remainder of the luteal phase of the cycle, the concentration of progesterone rose dramatically and was significantly higher than the control value (fig. 4.9).

The concentration of progesterone in the androstenedione-immunized/

immunized ewe sampled on day -1 (2.8 ng/ml) was lower than the level seen on day -5 (19.2 ng/ml) in this animal.

The concentrations of LH during the oestrous cycle in ewes immunized against A-11-BSA are shown in figures 4.12 and 4.13.

The mean basal level of LH in 2 out of 3 ewes sampled on day +1 was low (0.84 and 1.63 ng/ml) and was not significantly different from the corresponding control value (fig. 4.9). The concentration of LH in the third androstenedione-immunized ewe sampled on day +1 (7R-347, fig. 4.12) was very high (range: 18.8-85.0 ng/ml) and represented part of the preovulatory LH surge in this animal.

The levels of LH during the mid-luteal phase of the cycle (days -14 to -8) in the androstenedione-immunized ewes (figs. 4.12 and 4.13) were significantly higher than the corresponding control value (fig. 4.9).

The mean basal level of LH during the late-luteal phase (days -5 to -3) in the androstenedione-immunized ewes was lower than the levels found earlier in the luteal phase (fig. 4.9) but was still significantly higher than the corresponding control value (fig. 4.9).

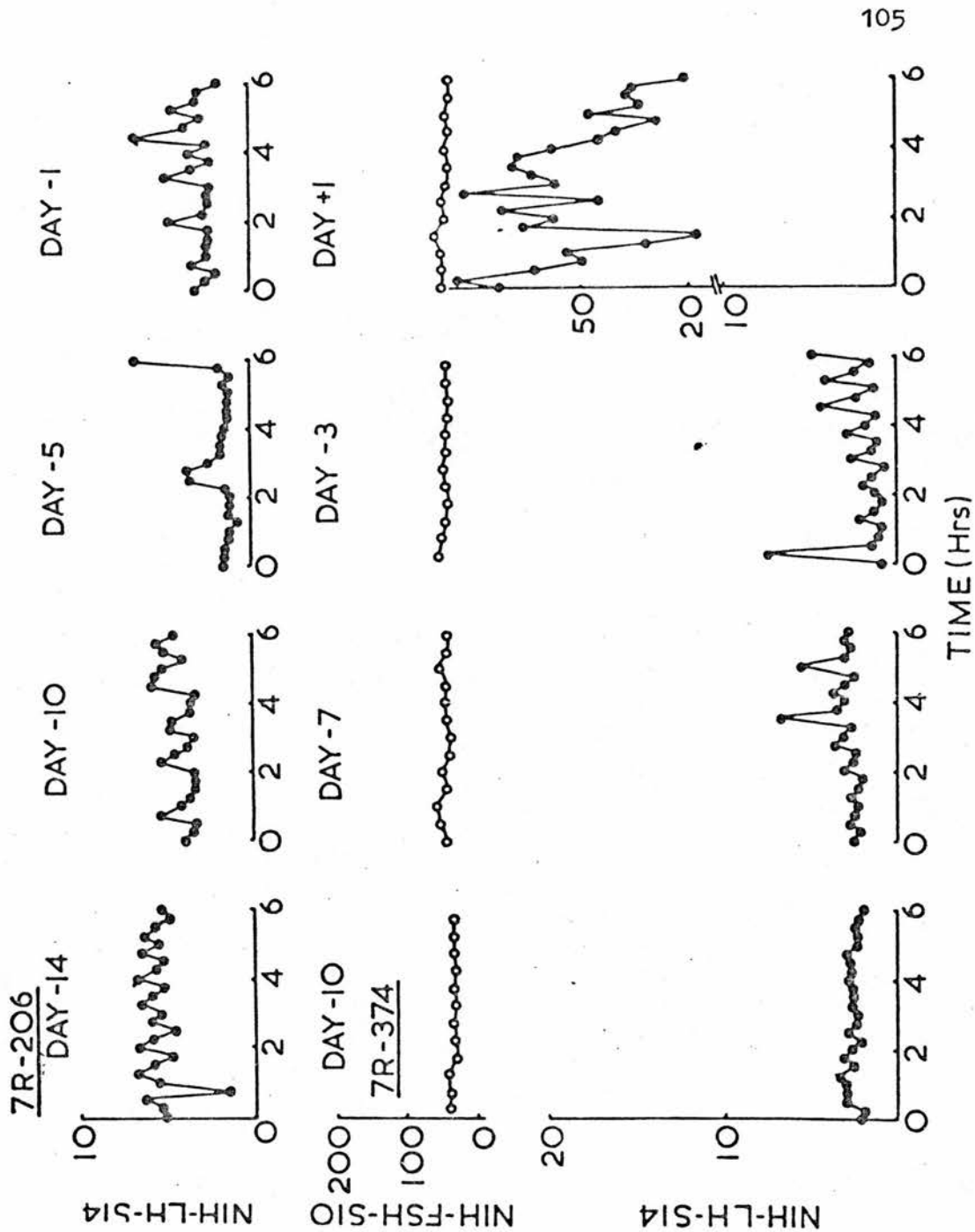
The concentrations of FSH measured in 3 androstenedione-immunized ewes during the oestrous cycle are also included in figures 4.12 and 4.13. During the cycle, the level of FSH was uniform within each animal. Notably, the animal with a preovulatory surge of LH on day +1 did not, however, show a corresponding elevation in the concentration of FSH (7R-374, fig. 4.12).

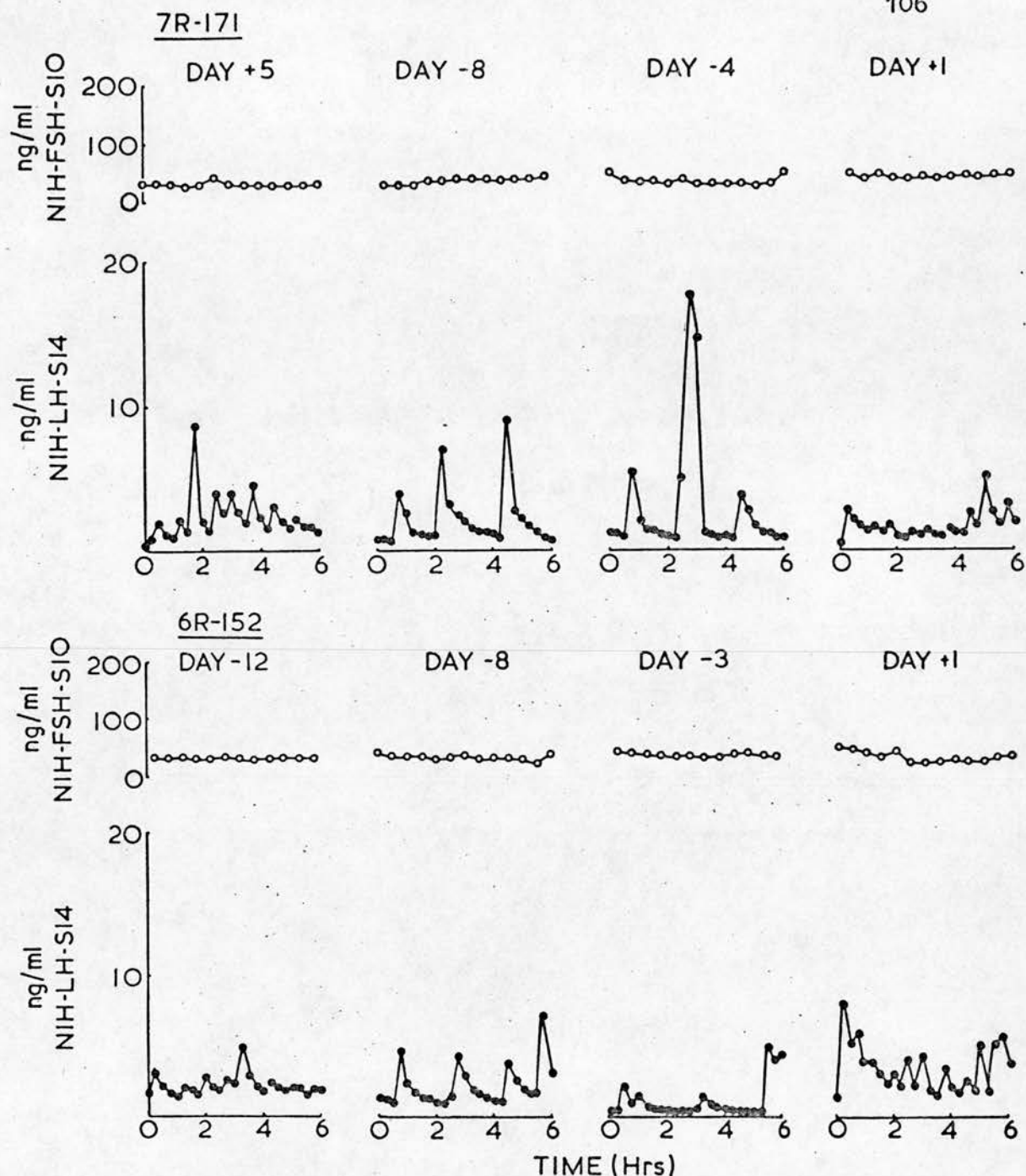
The mean concentration of FSH was significantly lower in the/

Figure 4.12.

The plasma concentrations of LH and FSH during the oestrous cycle in androstenedione-immunized ewes.

Samples were taken at 15-minute intervals for 6 hours at each sampling period. LH was assayed in every sample. FSH was measured in alternate samples.





**Figure 4.13** The plasma concentrations of LH and FSH in two androstenedione-immunized ewes during the oestrous cycle. Samples were taken at 15-minute intervals for 6 hours on each of 4 days during the oestrous cycle. LH was assayed in every sample. FSH was measured in alternate samples.

the androstenedione-immunized animals at all periods of the oestrous cycle examined (fig. 4.9).

The titres of steroid antibodies are shown in Table 4.4. Significant binding of androstenedione and testosterone, and, to a lesser extent, progesterone and oestrogens, was observed in the androstenedione-immunized ewes.

The binding of androstenedione, oestrone and testosterone was significantly higher than the steroid binding in control animals when determined by equilibrium dialysis (Table 4.5). A comparison of the steroid binding determined by each method yielded similar results to those described previously.

The concentrations of androstenedione, testosterone, oestradiol and oestrone in the jugular venous plasma of the control and androstenedione-immunized ewes are shown in Table 4.6. The concentration of all steroids in control plasma was low and neared the limit of sensitivity of the assays. The concentration of androstenedione in the androstenedione-immunized animals was significantly higher than the control value.

#### 4.3.3. DISCUSSION.

The oestrous cycle of the ewe is characterized by a short follicular phase lasting 3 to 4 days immediately prior to ovulation followed by a luteal phase of 13 to 14 days duration (Robinson, 1959).

The concentration of LH during the oestrous cycle is low apart from the preovulatory peak occurring near the end of each cycle (Geschwind & Dewey, 1968; Niswender et al. 1968; Reeves et al./

TABLE 4.4.  
THE TITRE<sup>a</sup> OF STEROID ANTIBODIES IN ANDROSTENEDIONE-IMMUNIZED EWES

CONTROL	<sup>3</sup> H-ANDROSTENEDIONE	<sup>3</sup> H-OESTRADIOL	<sup>3</sup> H-OESTRONE	<sup>3</sup> H-TESTOSTERONE	<sup>3</sup> H-PROGESTERONE
	N.S.	N.S.	N.S.	1:5	N.S.
ANDROSTENEDIONE- IMMUNIZED					
7R-206	1:400	1:3	1:11	1:36	1:30
7R-374	1:340	1:2	1:18	1:36	1:60
7R-171	1:210	1:2	1:7	1:64	1:58
6R-152	1:520	1:2	1:5	1:150	1:60

<sup>a</sup>Titre = The dilution of plasma which bound 50% of 15-20pg of tritiated steroid.

N.S. = <50% binding of the tritiated steroid at a dilution of 1:1.

TABLE 4.5.  
THE PERCENTAGE (M $\pm$ S.E.) OF 15-25pg OF TRITIATED STEROID BOUND BY A 1/5 DILUTION OF PLASMA  
FROM THE CONTROL AND ANDROSTENEDIONE-IMMUNIZED EWES DETERMINED BY EQUILIBRIUM DIALYSIS

	$^3$ H-ANDROSTENEDIONE	$^3$ H-OESTRADIOL	$^3$ H-OESTRONE	$^3$ H-TESTOSTERONE
CONTROL	55.7 $\pm$ 1.8 (n=5)	84.5 $\pm$ 1.4 (n=5)	73.8 $\pm$ 1.1 (n=5)	81.9 $\pm$ 2.2 (n=5)
ANDROSTENEDIONE- IMMUNIZED	99.4 $\pm$ 0.6* (n=4)	88.5 $\pm$ 1.2 (n=4)	98.9 $\pm$ 0.2* (n=4)	98.9 $\pm$ 0.2* (n=4)

\* p<0.001. Immunized vs. Control (Student's t-test).



TABLE 4.6.

THE CONCENTRATION OF ANDROSTENEDIONE, OESTRADIOL,  
OESTRONE AND TESTOSTERONE IN JUGULAR VENOUS PLASMA  
FROM THE CONTROL AND ANDROSTENEDIONE-IMMUNIZED EWES

	<u>ANDROSTENEDIONE</u> (ng/ml)	<u>OESTRADIOL</u> (ng/ml)	<u>OESTRONE</u> (ng/ml)	<u>TESTOSTERONE</u> (ng/ml)
<u>CONTROL</u>				
8R-354	0.095	0.002	0.006	0.074
8R-542	0.020	0.001	0.008	0.086
8R-602	0.036	0.006	0.011	0.103
8R-621	0.016	0.006	0.010	0.118
8R-676	0.054	0.003	0.009	0.048
Mean ( <u>+SE</u> )	0.044 <u>+0.014</u>	0.004 <u>+0.001</u>	0.009 <u>+0.001</u>	0.086 <u>+0.012</u>
<u>IMMUNIZED</u>				
6R-152	1.035	0.002	0.006	0.072
7R-206	2.258	0.002	0.069	0.113
7R-374	4.817	0.002	0.013	0.184
7R-171	2.659	0.005	0.006	0.100
Mean ( <u>+SE</u> )	2.692 <u>+0.780</u> *	0.003 <u>+0.001</u>	0.024 <u>+0.015</u>	0.117 <u>+0.024</u>

\*  $p < 0.01$  Immunized vs. Control (Student's t-test).



et al. 1970; Scaramuzzi et al. 1970b; Cunningham et al. 1975). These studies, however, were based on infrequent blood samples taken throughout the cycle. Since LH is secreted in a pulsatile manner, a true picture of LH secretion during the non-ovulatory periods of the cycle may not have been presented.

Relatively few studies have examined in detail the pattern of LH secretion during the oestrous cycle of the ewe apart from the periovulatory period. The present study examined the pattern of LH release by frequent sampling over a 6-hour period on each of 4 days spanning the luteal phase of the cycle. A large variation in the patterns of LH release between individual animals was found. In general, however, the basal level of LH was low (0.24-0.84 ng/ml) at all periods of the luteal phase examined although peaks in the concentrations of LH were observed. Fluctuations in the concentration of LH during the luteal phase of the cycle have been reported in other studies (Baird et al. 1976b; Foster, Jeffcoate, Crighton & Holland, 1976).

Secretory products from the ovary are thought to suppress the secretion of LH during the luteal phase of the cycle since ovariectomy during the breeding season results in a rapid rise in the level of LH (Roche, Foster, Karsch & Dzuik 1970b; Butler et al. 1972). Since oestradiol is also secreted by the follicles in the ovary during the luteal phase of the cycle (Baird & Scaramuzzi, 1976a) it is generally thought that this steroid exerts a negative feedback action on the hypothalamo-hypophyseal axis.

Progesterone is secreted in large amounts by corpus luteum/

luteum of the ovary during the luteal phase of the cycle (Short et al. 1963; Moore, Barrett, Brown, Schindler, Smith & Smyth, 1969; Baird et al. 1976a). Progesterone will block the positive feedback action of oestradiol on LH release in ovariectomized (Goding, Blockey, Brown, Catt & Cumming, 1970; Scaramuzzi et al. 1971) and intact (Baird & Scaramuzzi, 1976b) ewes. Furthermore, oestradiol is effective in inducing a discharge of LH and subsequent ovulation in intact and hysterectomized ewes when given on day 3 of the cycle (Bolt, Kelley & Hawk, 1971), a time when circulating progesterone has not reached maximal levels (Thorburn, Bassett & Smith, 1969; Baird et al. 1976a). The same treatment on day 10 of the cycle, when progesterone has reached maximal levels in the peripheral plasma (Thorburn et al. 1969; Baird et al. 1976a), failed to induce an LH discharge or ovulation (Bolt et al. 1971). It is likely, therefore, that progesterone might well prevent premature preovulatory discharges of LH during the luteal phase of the cycle.

A negative feedback action of progesterone on LH secretion during the luteal phase of the cycle has been more difficult to evaluate. The effect of progesterone on the level of LH in ovariectomized ewes has yielded equivocal results. Goding et al. (1970) and Scaramuzzi et al. (1971) found that progesterone had no effect on the level of LH in long-term ovariectomized ewes. On the other hand, Davis and Borger (1974) showed that the level of LH was lowered in ovariectomized ewes bearing implants of progesterone. It has recently been demonstrated that progesterone implants will prevent the immediate post-ovariectomy elevation of LH in immature lambs (Foster/

(Foster & Karsch, 1976) and adult ewes (Karsch et al. 1976).

The concentration of LH was suppressed to a greater degree during anoestrus than during the breeding season in ovariectomized ewes bearing oestradiol implants (Karsch et al. 1976), suggesting <sup>that</sup> the sensitivity of the hypothalamo-hypophyseal axis towards oestradiol is reduced during the breeding season. It is conceivable that progesterone could increase the sensitivity of the hypothalamo-hypophyseal axis towards the negative feedback effects of oestradiol during the luteal phase of the oestrous cycle.

The secretion of FSH during the oestrous cycle of the ewe has received only limited attention. A simultaneous peak in the plasma concentrations of LH and FSH occurs prior to ovulation (L'Hermite et al. 1972; Pant, Fitzpatrick & Hopkinson, 1973; Salamonsen et al. 1973; Cunningham et al. 1975). A second peak of FSH on the day after oestrus has been reported in some studies (L'Hermite et al. 1972; Pant et al. 1973). The concentration of FSH showed marked fluctuations during the remainder of the cycle (Salamonsen et al. 1973; Cunningham et al. 1975; McNeilly et al. 1976).

The concentrations of FSH in the control animals in the present study showed little variation either within the sampling period or between periods of the cycle in individual animals. The concentrations, however, were within the range of values reported in other studies (L'Hermite et al. 1972; Salamonsen et al. 1973; McNeilly et al. 1976).

The concentrations of progesterone determined during the oestrous cycle in the control animals were within the same range, and followed a similar pattern to those reported in earlier studies (Thorburn et al. 1969; McNatty, Revfiem & Young/

Young, 1973).

The neutralization of biologically active androstenedione produced an elevation in the concentration of LH during the luteal phase of the cycle. These findings would suggest that androstenedione, either directly, or through its metabolites, could modify the negative feedback action of oestradiol.

The concentration of FSH in the androstenedione-immunized ewes during the oestrous cycle was significantly lower than the control value. These findings confirm a similar finding seen in androstenedione-immunized ewes during anoestrus.

The marked elevation in the concentration of progesterone can best be explained by the presence of antibodies to progesterone. Such antibodies could have reduced the clearance of progesterone from the circulation (Schwartz et al. 1975). However, the concentration of progesterone was not elevated in female rhesus monkeys actively immunized against progesterone (Schwartz et al. 1975).

It is tempting to suggest that the elevated level of LH during the luteal phase of the cycle had stimulated the synthesis and secretion of progesterone by the corpus luteum. It is well established that LH will increase the synthesis of progesterone by luteal tissue in vitro (Savard, Marsh & Rice, 1965; Kaltenbach, Cook, Niswender & Nalbandov, 1967). However, in vivo studies in sheep have shown that LH will produce only a transient increase in the secretion of progesterone (Short et al. 1963; Collett, Land & Baird, 1973) perhaps due to a depletion of cholesterol in luteal tissue thereby developing a state of refractoriness towards further stimulation (Baird/

(Baird & Collett, 1973). However, these studies were performed with relatively high levels of LH (100  $\mu$ g/h to 1000  $\mu$ g/h). It is possible that the slightly elevated levels of LH present in the androstenedione-immunized animals may have produced a sustained increase in the secretion of progesterone. This event in conjunction with the presence of progesterone antibodies could well have led to the elevated level of progesterone observed in the androstenedione-immunized ewes.

It is interesting to note that a reduction in the level of LH occurred in the late-luteal phase of the cycle in the androstenedione-immunized ewes. It is possible that the very high level of progesterone present at this time may have resulted in a negative feedback effect as suggested by Karsch et al. (1976). It is equally possible that the secretion of oestradiol was increased at this time, although this parameter was not measured, thus resulting in an increased negative feedback action on LH secretion.

The time interval between the onset of oestrus and the preovulatory LH peak was delayed in the androstenedione-immunized ewes ( $M \pm S.E.$ ; control =  $7.2 \pm 0.8$  hours,  $n=5$ ; androstenedione-immunized =  $22.7 \pm 4.8$  hours,  $n=3$ ) (R.J. Scaramuzzi, Unpublished results). This finding suggests that the positive feedback action of oestradiol during the oestrous cycle is also modified by androstenedione. However, the presence of a significant amount of progesterone (2.8 ng/ml) on the day before oestrus in one androstenedione-immunized ewe may have prevented the positive feedback action of oestradiol as discussed earlier.

The titre of androstenedione and testosterone antibodies  
in/

in the androstenedione-immunized ewes during the oestrous cycle (45 days after a booster immunization) was equivalent to, or less than the lowest titres observed in these animals during the anoestrous experiment (14 days after a booster immunization). It is perhaps surprising that any alteration in the gonadotrophin patterns was observed during the oestrous cycle since little alteration in the release of LH or FSH was found during anoestrus in the animals with the lowest titres. Since the hypothalamo-hypophyseal sensitivity to the negative feedback effects of oestradiol is less during the breeding season than during anoestrus (Karsch et al. 1976), it is possible that the modulatory role of androstenedione or its metabolites during the breeding season may have assumed greater importance in the maintenance of negative feedback during this period of reduced sensitivity to oestradiol.

#### 4.4. THE OVARIAN MORPHOLOGY AND STEROID CONCENTRATIONS IN EWES ACTIVELY IMMUNIZED AGAINST A-11-BSA.

##### 4.4.1. PROCEDURE.

The present experiment was performed in collaboration with Drs. D.T. Baird and P.F.A. Van Look, and Mr. W. Davidson.

The nine control and androstenedione-immunized ewes were laparotomized during the luteal phase (between days 7 and 12) of the oestrous cycle, 156-179 days after the fourth booster immunization.

The laparotomy procedure followed the method described by/

by Baird and Scaramuzzi (1976a).

Anaesthesia was induced by an intravenous injection of sodium thiopentone. An endotracheal tube was inserted and anaesthesia maintained with halothane (1-3% in oxygen).

The ovaries were exposed through a mid-line lower abdominal incision. The number and diameter of corpora lutea and large follicles ( $\geq 5$ mm diameter) were recorded. The utero-ovarian vein on both sides was cannulated and the blood vessels draining the uterus ligated as previously described (Mattner & Thorburn, 1969; Baird & Scaramuzzi, 1976a). The animal was heparinized with 10,000 I.U. Heparin (i.v.) and a sample of ovarian venous blood (10-25ml) collected from each ovary. A sample of jugular venous blood was obtained by venipuncture at the same time as the ovarian collections. The blood samples were centrifuged and the plasma stored at  $-20^{\circ}\text{C}$ .

After completion of the blood sampling, the ovaries and adrenal glands were removed. The corpora lutea were excised from the remainder of the ovary. The tissues were weighed and ovarian tissue placed in Bouin's fixative and the adrenals in Helly's fixative. After 24-36 hours, the tissues were removed from the fixatives. The adrenals were washed thoroughly with distilled water and all tissues were stored in 70% ethanol in water (v/v) for subsequent histological examination.

The animals were killed at the completion of the experiment without regaining consciousness by an overdose of anaesthetic.

The titre of steroid antibodies and the percentage of steroid/



steroid bound after equilibrium dialysis were determined in the sample of jugular venous plasma obtained from each animal. The concentrations of endogenous progesterone, oestradiol, oestrone, androstenedione and testosterone were measured in both the peripheral (jugular venous) and ovarian venous plasma samples from all animals.

#### 4.4.2. RESULTS.

The steroid antibody titres, determined by charcoal absorption, in plasma from the control and androstenedione-immunized ewes are shown in Table 4.7. Since the experiment was performed 156-179 days after a booster immunization, the titres of androstenedione antibodies in the androstenedione-immunized ewes, although higher than the control value, were low, ranging from 1:100 to 1:150. A significant titre of testosterone antibodies was found in two androstenedione-immunized ewes (7R-171 - 1:450; 6R-152 - 1:400). The binding of both oestradiol and oestrone was similar in the control and androstenedione-immunized animals (Table 4.7).

Although low steroid antibody titres were found in the androstenedione-immunized ewes, the percentage of androstenedione and testosterone bound by plasma from these ewes after equilibrium dialysis was significantly higher than the corresponding control values (Table 4.8) reaching almost 100%. The binding of oestrone (91.3%) was significantly higher than the control value. No difference was found in the binding of oestradiol by plasma from the control and androstenedione-immunized animals (Table 4.8).



TABLE 4.7.

THE TITRE<sup>a</sup> OF STEROID ANTIBODIES IN ANDROSTENEDIONE-IMMUNIZED EWES

<u>CONTROL</u>	<u><sup>3</sup>H-ANDROSTENEDIONE</u>	<u><sup>3</sup>H-OESTRADIOL</u>	<u><sup>3</sup>H-OESTRONE</u>	<u><sup>3</sup>H-TESTOSTERONE</u>
	N.S.	N.S.	N.S.	1:2

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<u>ANDROSTENEDIONE- IMMUNIZED</u>				
7R-171	1:100	N.S.	N.S.	1:450
7R-206	1:130	N.S.	1:1	1:6
6R-152	1:100	N.S.	N.S.	1:400
7R-374	1:100	N.S.	N.S.	N.S.

<sup>a</sup>Titre = The initial dilution of plasma which bound 50% of 15-20pg of tritiated steroid.

N.S. = &lt;50% binding of the tritiated steroid at a dilution of 1:1.

TABLE 4.8.  
THE PERCENTAGE (N<sup>±</sup>S.E.) OF 15-25pg OF TRITIATED STEROID BOUND BY A 1/5 DILUTION OF PLASMA  
FROM THE CONTROL AND ANDROSTENEDIONE-IMMUNIZED EGGS DETERMINED BY EQUILIBRIUM DIALYSIS

	<sup>3</sup> H-ANDROSTENEDIONE	<sup>3</sup> H-OESTRADIOL	<sup>3</sup> H-OESTRONE	<sup>3</sup> H-TESTOSTERONE
CONTROL	56.2±2.1 (n=4)	89.2±1.8 (n=4)	79.3±0.8 (n=4)	85.6±1.4 (n=4)
ANDROSTENEDIONE- IMMUNIZED	99.2±0.1* (n=4)	97.1±0.5 (n=4)	91.3±2.0* (n=4)	98.3±0.5* (n=4)

\* p<0.001. Immunized vs. Control (Student's t-test).

The concentration of oestradiol, oestrone, androstenedione and testosterone in peripheral and ovarian venous plasma from the control and androstenedione-immunized ewes are shown in Tables 4.9 and 4.10 respectively.

The concentration of androstenedione in both peripheral and ovarian venous plasma from the androstenedione-immunized ewes was significantly higher than the corresponding control values (Table 4.10). Although the ovarian blood flow was not determined, thus preventing an estimation of the ovarian steroid secretion rates, the fact that the level of androstenedione in ovarian venous plasma from the androstenedione-immunized ewes was consistently higher than the corresponding peripheral concentration (Table 4.10) suggested that the ovarian secretion of this steroid had been increased.

The androstenedione-immunized ewe with the highest testosterone antibody titre also showed an elevation in the concentration of testosterone in peripheral and ovarian venous plasma (7R-171, Table 4.10). In the remaining 3 androstenedione-immunized ewes, the concentration of testosterone in peripheral and ovarian venous plasma was not significantly different from the control values.

The level of oestrone in ovarian venous plasma from the androstenedione-immunized ewes was similar to the corresponding control value (Table 4.10). The concentration of oestrone in the peripheral plasma from two androstenedione-immunized animals (6R-152, 7R-206, Table 4.10) was significantly higher than the control value, suggesting that the presence of oestrone antibodies in these animals had slightly/

TABLE 4.9.  
THE CONCENTRATION OF OESTRADIOL, OESTRONE, ANDROSTENEDIONE AND TESTOSTERONE  
IN PERIPHERAL AND OVARIAN VENOUS PLASMA FROM CONTROL EWES

	Oestradiol(ng/ml)		Oestrone(ng/ml)		Androstenedione(ng/ml)		Testosterone(ng/ml)	
	P	LO	P	LO	P	LO	P	LO
8R-542	0.039	0.113	0.024	0.150	0.113	0.473	0.034	0.205
8R-676	0.036	N.S.	0.021	N.S.	0.331	N.S.	0.089	N.S.
8R-621	0.021	0.029	0.015	0.058	0.020	0.427	0.027	0.058
8R-602	0.036	0.058	0.015	0.085	0.037	0.142	0.026	0.132
Mean	0.033	0.052	0.019	0.096	0.125	0.315	0.044	0.186
S.D.	0.008	0.032	0.005	0.043	0.143	0.151	0.030	0.132

P = peripheral. LO = left ovary. RO = right ovary.

N.S. = no sample.

TABLE 4.10.

THE CONCENTRATION OF OESTRADIOL, OESTRONE, ANDROSTENEDIONE AND TESTOSTERONE  
IN PERIPHERAL AND OVARIAN VENOUS PLASMA FROM ANDROSTENEDIONE-IMMUNIZED EWES.

	Oestradiol (ng/ml)			Oestrone (ng/ml)			Androstenedione (ng/ml)			Testosterone (ng/ml)		
	P	RO	LO	P	RO	LO	P	RO	LO	P	RO	LO
<u>IMMUNIZED</u>												
7R-171	0.021	0.036	0.024	0.021	0.080	0.152	8.4*	21.1*	12.5*	0.287*	0.673*	0.509*
6R-152	0.034	0.025	0.040	0.037*	0.121	0.133	2.8*	8.9*	9.2*	0.093	0.235	0.384
7R-374	0.034	0.078	0.043	0.026	0.091	0.064	3.9*	10.0*	8.2*	0.055	0.403	0.176
7R-206	0.027	0.038	0.210*	0.039*	0.101	0.129	5.0*	14.6*	14.8*	0.078	0.298	0.406
<u>CONTROL</u>	0.033	0.052		0.019	0.096		0.125	0.315		0.044	0.186	
(M±SD)	±0.008	±0.032		±0.005	±0.043		±0.143	±0.151		±0.030	±0.132	

P = peripheral. RO = right ovary. LO = left ovary.

\* Value lies outside corresponding control mean ± 2 S.D.

slightly prolonged the clearance rate of oestrone from the circulation.

No difference in the concentration of oestradiol in peripheral or ovarian venous plasma was found between the control and androstenedione-immunized ewes (Table 4.10).

The concentration of progesterone in peripheral and ovarian venous plasma from control and androstenedione-immunized animals are shown in Tables 4.11 and 4.12 respectively.

The peripheral levels of progesterone in the control animals (Table 4.11) are similar to those found during the normal luteal phase of the oestrous cycle. The concentration of progesterone in the ovarian venous plasma in control ewes was related to the presence or absence of corpora lutea. The progesterone level was far greater in ovarian venous plasma from the ovary containing one or more corpora lutea than the contra-lateral ovary without corpora lutea (Table 4.11).

The concentration of progesterone in peripheral plasma from the androstenedione-immunized ewes was significantly higher than the corresponding control value (Table 4.12). However, the level of progesterone in ovarian venous plasma from the ovary containing corpora lutea was similar in both control and androstenedione-immunized animals.

The weight of ovarian tissue together with the number of large ovarian follicles ( $\geq 5$ mm diameter) in control and androstenedione-immunized ewes are shown in Tables 4.13 and 4.14 respectively.

Although the weight of luteal tissue was similar in both the control and androstenedione-immunized ewes, the weight/

TABLE 4.11.

THE CONCENTRATION OF PROGESTERONE IN PERIPHERAL AND  
OVARIAN VENOUS PLASMA FROM CONTROL EWES.

	<u>Proges terone(Ng/ml)</u>		
	P	LO	RO
8R-542	2.7	974(2)	N.S.(0)
8R-676	2.9	N.S.(0)	403(1)
8R-621	2.9	8.1(0)	460(2)
8R-602	3.8	492(1)	3.9(0)

P = peripheral. LO = left ovary. RO = right ovary

N.S. = no sample.

( ) = number of corpora lutea.



TABLE 4.12.  
THE CONCENTRATION OF PROGESTERONE IN PERIPHERAL AND  
OVARIAN VENOUS PLASMA FROM ANDROSTENEDIONE-  
IMMUNIZED EWES.

	<u>Proges terone (ng/ml)</u>		
	P	RO	LO
<u>IMMUNIZED</u>			
7R-171	22.1*	1006(2)	29.3(0)
6R-152	31.7*	116(0)	683(2)
7R-374	19.2*	820(1)	465(1)
7R-206	32.2*	830(1)	786(1)
<u>CONTROL</u>			
P ( <u>M</u> ±SD)	3.1±0.5		
Ovary without C.L. ( <u>M</u> ±SD)		3.1±3.8	
n.		4	
Ovary with C.L. ( <u>M</u> ±SD)		582±263	
n.		4	

P = peripheral. RO = right ovary. LO = left ovary.

C.L. = corpora lutea.

n. = number of observations.

( ) = number of C.L.

TABLE 4.13.  
THE OVARY IN CONTROL EWES

		Day of the cycle	Wt.C.L. (mg)	Wt.NLT (mg)	No. of follicles Diameter (mm)	
					5-11	11-15
8R-542	RO	11	-	632	0	0
	LO		492,556	863	1	0
8R-676	RO	9	598	981	0	0
	LO		-	1255	1	0
8R-621	RO	10	403,301	930	0	0
	LO		-	730	0	0
8R-602	RO	10	-	551	0	0
	LO		631	666	2	0
Mean			497	826	1.0 $\pm$ 0.8	0
S.D.			126	229	(no. per animal)	

C.L. = corpora lutea. NLT = non-luteal tissue - ovary  
after the removal of C.L.

RO = right ovary. LO = left ovary.

TABLE 4.14  
THE OVARY IN ANDROSTENEDIONE-IMMUNIZED EWES

		Day of the cycle	Wt.C.L. (mg)	Wt.NLT (mg)	No. of follicles Diameter(mm)	
					5-11	>11
<u>IMMUNIZED</u>						
7R-171	RO	9	548,493	2925*	1	1
	LO		-	1265	0	0
6R-152	RO	9	-	1948*	1	0
	LO		703,670	1563*	2	0
7R-374	RO	8	521	1342*	2	0
	LO		472	1318*	1	0
7R-206	RO	11	807	2324*	0	1
	LO		694	1529*	1	0
<hr/>						
CONTROL			497	826	1.0 $\pm$ 0.8	0
(M $\pm$ SD)			126	228	(no.per animal)	
n.			6	8	4	

C.L. = corpora lutea. NLT = non-luteal tissue - ovary after the removal of C.L. RO = right ovary. LO = left ovary.

n = number of observations.

\* Value lies outside the corresponding control mean  $\pm$  2 S.D.

weight of non-luteal ovarian tissue (the remainder of the ovary after the removal of corpora lutea) was significantly higher in the androstenedione-immunized ewes (Table 4.14).

The number of large follicles per animal was 2-3 times higher in the androstenedione-immunized ewes compared to the control animals (Table 4.14.).

Similarly, the ovulation rate, determined by the number of corpora lutea per animal, was higher in the androstenedione-immunized ewes ( $2.0 \pm 0.0$ ;  $M \pm S.E.$ ,  $n=4$ ) compared to the ovulation rate in the control animals ( $1.5 \pm 0.3$ ;  $M \pm S.E.$ ,  $n=4$ ), although the difference was not statistically significant ( $p > 0.05$ , Student's t-test).

The weight of the adrenal glands was similar in both the androstenedione-immunized ( $1.93 \pm 0.90$ ;  $M \pm S.E.$ ,  $n=6$ ) and control ( $1.71 \pm 0.30$ ;  $M \pm S.E.$ ,  $n=8$ ) animals.

The results of the histological examination of the ovaries were incomplete at the time of writing.

#### 4.4.3. DISCUSSION.

The elevated level of androstenedione in the peripheral plasma of ewes actively immunized against A-11-BSA indicated the presence of high affinity antibodies to androstenedione which served to reduce the clearance rate of this steroid from the circulation. A similar elevation in the peripheral concentrations of oestradiol and testosterone have been found in rats and rabbits actively immunized against the homologous steroid-protein conjugate (Hiller et al. 1973b, 1975a,b; Nieschlag et al. 1973, 1974).

Similarly/

Similarly, the presence of elevated levels of progesterone in peripheral plasma from the androstenedione-immunized ewes may have arisen from the presence of progesterone antibodies.

The fact that the concentration of androstenedione in ovarian venous plasma from the androstenedione-immunized ewes was  $1\frac{1}{2}$ -3 times higher than the corresponding peripheral level suggested that the ovarian secretion of this steroid had been increased. Since the principal source of androstenedione is the ovarian follicle (Baird & Scaramuzzi, 1976a) the increase in the weight of the non-luteal ovarian tissue in the androstenedione-immunized ewes further suggested an elevation in the steroid secretion by this compartment of the ovary.

It has been demonstrated that LH will increase the secretion rate of both oestradiol and androstenedione by the ovary transplanted to the neck (McCracken et al. 1969; Baird et al. 1976b). It is likely, therefore, that the elevated levels of LH during the oestrous cycle in the androstenedione-immunized ewes had stimulated ovarian steroid secretion.

It is somewhat surprising that the concentration of oestradiol in ovarian venous plasma from the androstenedione-immunized ewes was not also increased in response to the elevated levels of LH. It has been proposed that oestradiol production by the follicle arises in part by androstenedione of thecal origin entering the granulosa cell where aromatization to oestrogens can occur (Short, 1964; Younglai & Short, 1970). Since immunoglobulins can enter the follicular fluid (Edwards, 1974), it is possible that the presence of androstenedione antibodies may have effectively reduced the availability of androstenedione, a major precursor of oestradiol (Rado et al. 1970/

1970), thereby preventing an increase in oestradiol secretion.

It has recently been demonstrated that FSH, rather than LH, will stimulate the aromatization of testosterone to oestradiol in vitro by the rat ovary (Moon, Dorrington & Armstrong, 1975) or by rat granulosa cells (Dorrington, Moon & Armstrong, 1976). It is possible, therefore, that the reduction in the level of FSH in the androstenedione-immunized ewes may have reduced the degree of androgen aromatization within the follicle thereby preventing the increase in oestradiol secretion.

The answer to the lack of elevated oestradiol secretion in the androstenedione-immunized animals may well lie in either, or both the neutralization of an oestradiol precursor or a reduction in the degree of aromatization within the ovary.

It has generally been accepted that FSH plays an important role in follicular development. However, the number of large follicles in the ovaries of the androstenedione-immunized ewes was increased even though the level of FSH was lower than that found in the control animals. In the ewe, several waves of follicular growth are thought to occur during the oestrous cycle (Smeaton & Robertson, 1971; Brand & De Jong, 1973). Such follicles either undergo atresia or, in the presence of the appropriate stimuli, ovulate and are luteinized. It is possible that the altered follicular environment and abnormal gonadotrophin levels in the androstenedione-immunized ewes may, in some way, have altered the rate of follicular atresia. This question, however, cannot be answered satisfactorily until the histological examination of the ovaries from the androstenedione-immunized animals becomes available.

The ovulation rate in the androstenedione-immunized ewes was higher/

higher than the control value. Although the difference in ovulation rate in the control and androstenedione-immunized animals was not statistically significant in the present experiment, perhaps due to the length of time since the booster immunization (156-179 days), laparotomy of these same animals 62-70 days after the third booster immunization revealed a significantly higher ovulation rate in the androstenedione-immunized ewes (androstenedione-immunized ewes:  $2.25 \pm 0.2$ ;  $M \pm S.E.$ ,  $n=4$ ; control:  $1.0 \pm 0.0$ ;  $M \pm S.E.$ ,  $n=5$ ) (R.J. Scaramuzzi, Unpublished results).

The reason for the increase in the ovulation rate in the androstenedione-immunized ewes is not clear. It is possible that the longer interval between the onset of behavioural oestrus and the preovulatory LH surge in the androstenedione-immunized ewes may have allowed more follicles to reach the preovulatory stage. Alternatively, a reduced rate of follicular atresia may have increased the number of preovulatory follicles able to respond to the ovulatory stimuli.

It was thought that the slight increase in ovulation rate in the androstenedione-immunized ewes might have a commercial application for increasing the fertility of domestic animals. To this end, a patent application has been filed\*.

To further examine the possibility of increased fertility in sheep actively immunized against A-11-BSA, a group of 18 Welsh mountain ewes was immunized with 1mg of A-11-BSA during August 1976 (Van Look, Clarke, Davidson & Scaramuzzi, 1976. In preparation/

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\* N.D. Martensz, R.J. Scaramuzzi and P.F.A. Van Look: "Improvements in or relating to animal breeding techniques". U.K. Patent application No. 32672/75.



preparation). Another 7 ewes received 1mg of BSA and served as controls. A booster immunization of 1mg of antigen was given to all animals 12 weeks after the primary immunization.

One month later, the ewes were laparotomized. The ovulation rate in androstenedione-immunized ewes ( $2.3 \pm 0.3$ ;  $M \pm S.E.$ ,  $n=18$ ) was significantly higher ( $p < 0.05$ , Student's t-test) than the ovulation rate in the control animals ( $1.6 \pm 0.2$ ;  $M \pm S.E.$ ,  $n=7$ ).

The 7 control ewes and 17 androstenedione-immunized animals (1 animal had died as a result of the laparotomy) were mated at the end of January and during February 1976, with 3 Welsh mountain rams of proven fertility. All the control animals mated successfully on the first exposure to the rams. The mating rate, however, was reduced in the experimental group and 6 ewes returned to the ram once, 3 ewes returned twice and 2 ewes returned 3 times.

The subsequent pregnancies were allowed to proceed to term. The lambing results are shown in Table 4.15. It is immediately evident that although the number of twin births was increased in the androstenedione-immunized ewes, the number of ewes delivered in this group (7/16) was well beneath the delivery rate in the control animals (6/7).

The reason for the reduced conception rate in the androstenedione-immunized ewes is not clear. Work is currently in progress to examine the fertility in a larger number of animals (R.J. Scaramuzzi, C.S.I.R.O. Division of Animal Research, Prospect, N.S.W., Australia).

TABLE 4.15THE FERTILITY IN ANDROSTENEDIONE-IMMUNIZED EWES.

	<u>CONTROL</u>	<u>ANDROSTENEDIONE- IMMUNIZED</u>
Number of Ewes	7	15
Number of ewes delivered	6	8
Number of lambs born:		
Singleton .. .. .	4	3
Twins .. .. .	2	5
Number of lambs surviving	8	11*
Sex ratio of surviving lambs (female/male)	1/1	10/1

\* 1 intra-uterine death; 1 death due to neonatal asphyxia.

4.5. CHAPTER SUMMARY

(1) The presence of antibodies to androstenedione, and, to a lesser extent, testosterone and oestrone was demonstrated in jugular venous plasma from ewes actively immunized against A-11-BSA.

(2) During anoestrus, the frequency of spontaneous LH discharges was increased in the androstenedione-immunized ewes. In contrast, the concentration of FSH was reduced in animals with high androstenedione antibody titres. The positive feedback response of LH and FSH to 25 $\mu$ g of oestradiol benzoate was delayed or absent in ewes with high antibody titres.

(3) The level of LH was elevated during the luteal phase of the oestrous cycle in the androstenedione-immunized ewes. In contrast, a reduction in the concentration of FSH occurred in these animals.

(4) It is postulated that androstenedione, either directly, or through its extra-ovarian conversion to oestrone, may serve to modulate the negative and positive feedback actions of oestradiol in the control of LH secretion in the ewe.

(5) The reduction in the level of FSH in the absence of circulating biologically active androstenedione was difficult to explain. This phenomenon may have been due to the antagonism of the negative feedback action of oestradiol by androstenedione.

(6) The concentration of androstenedione in peripheral plasma was significantly higher in the androstenedione-immunized ewes. This finding was attributed to a decreased clearance rate/

rate of this steroid from the circulation due to the presence of high-affinity antibody binding. A similar conclusion may also account for the presence of elevated levels of progesterone during the luteal phase of the oestrous cycle in the androstenedione-immunized animals.

(7) The finding that the concentration of androstenedione in ovarian venous plasma in the androstenedione-immunized ewes was  $1\frac{1}{2}$ -3 times greater than the corresponding level in peripheral plasma suggested that the ovarian secretion of this steroid had been increased. This finding was further corroborated by an increase in the weight of non-luteal ovarian tissue in the androstenedione-immunized animals.

(8) The presence of normal oestradiol levels in ovarian venous plasma in the androstenedione-immunized ewes may be attributed to a reduced availability of the major precursor of oestradiol and/or a reduction in the degree of aromatization of androgens to oestrogens in these animals due to the lower level of FSH.

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CHAPTER 5.

THE EFFECTS OF ACTIVE IMMUNIZATION AGAINST  
17 $\beta$ -OESTRADIOL-6-(O-CARBOXYMETHYL)OXIME-BOVINE SERUM  
ALBUMIN ON THE RELEASE OF LH AND FSH AND THE  
CONCENTRATION OF STEROIDS IN THE EWE.

### 5.1. INTRODUCTION

Oestradiol-17 $\beta$  (oestradiol) is the major oestrogen secreted by the ovary in the sheep (Short et al. 1963; Lindner et al. 1964; Baird et al. 1968b, 1973).

The source of oestradiol is thought to be the ovarian follicle since the concentration of oestradiol is higher in ovarian venous blood draining the ovary with medium or large follicles than the venous drainage from the contralateral ovary (Moore et al. 1969; Bjersing, Hay, Kann, Moor, Naftolin, Scaramuzzi, Short & Younglai, 1970; Baird & Scaramuzzi, 1976a) and enucleation of the large follicle causes a marked reduction in the secretion of oestradiol (Baird & Scaramuzzi, 1976a).

The biosynthetic pathway of oestradiol within the follicle is thought to be via the " $\Delta^4$ -pathway" (fig.5.1) (Short, 1964; Younglai & Short, 1970) and probably involves both the theca and granulosa cells (Short, 1962, 1964; Younglai & Short, 1970).

Gonadotrophins have been shown to stimulate the secretion of oestradiol. LH, in particular, will increase the secretion rate of oestradiol by the autotransplanted ovary (McCracken et al. 1969). It is generally accepted that LH acts by increasing the conversion of cholesterol to pregnenolone and progesterone (Savard et al. 1965; Flint & Armstrong, 1972). Recent evidence suggests that FSH may serve to stimulate the aromatization of the androgen precursors of oestrogens (Moon et al. 1975; Armstrong & Papkoff, 1976; Dorrington et al. 1976).

The/

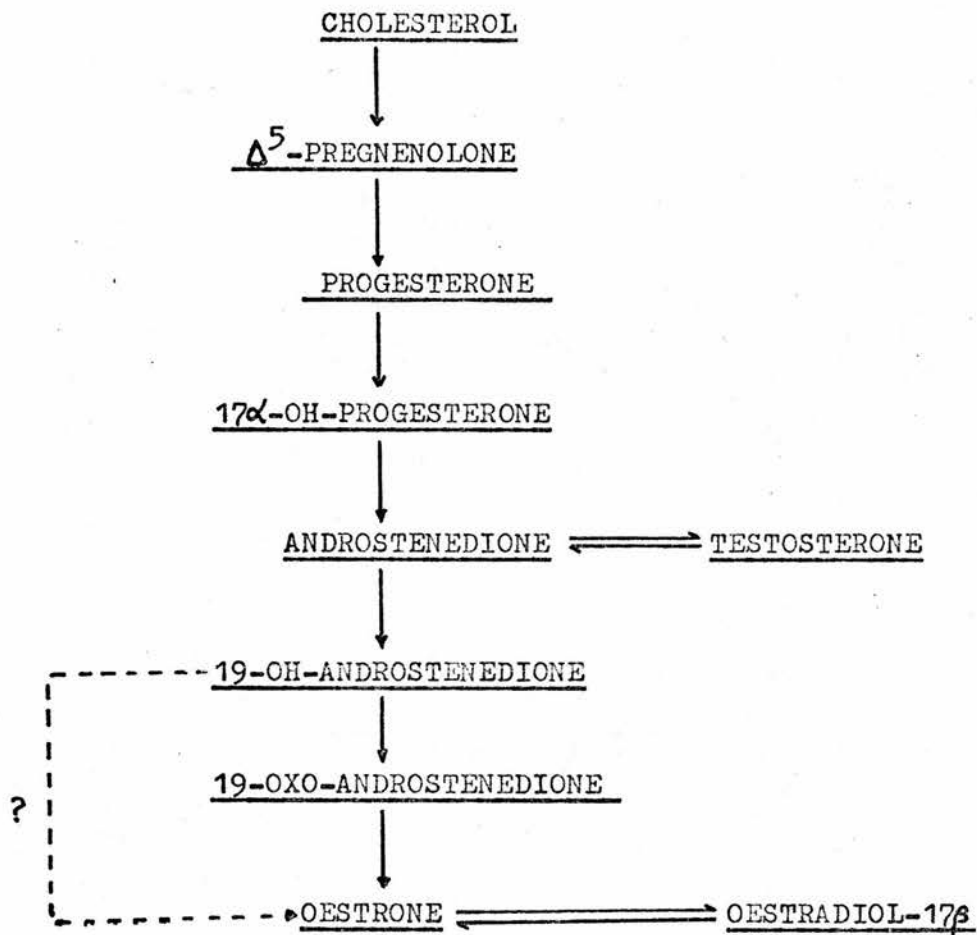


Figure 5.1. The " $\Delta^4$ -pathway" of oestrogen biosynthesis in the ovarian follicle.



The secretion of oestradiol has been studied throughout the oestrous cycle in sheep. Most notably, a rise in the secretion rate of oestradiol occurs towards the end of the cycle, beginning about 3 days before oestrus and reaching maximal levels about 20-30 hours before the onset of oestrus (Moor et al. 1969; Scaramuzzi et al. 1970b; Cox, Mattner & Thorburn, 1971; Bjersing et al. 1972; Baird et al. 1976a). At the same time, the level of progesterone is falling (Moore et al. 1969; Cox et al. 1971; Baird et al. 1976a) due to the regression of the corpus luteum (Bjersing et al. 1972). It has been demonstrated that oestradiol will induce oestrus in progesterone-primed ovariectomized ewes (Scaramuzzi et al. 1971), suggesting that the rise in oestradiol towards the end of the cycle may be responsible for the expression of oestrous behaviour in the intact cycling ewe. Furthermore, the fact that oestradiol will induce a surge of LH in ovariectomized (Radford, Wheatley & Wallace, 1969; Goding et al. 1970; Scaramuzzi et al. 1971) and anoestrous (Goding et al. 1969; Beck & Reeves, 1973) ewes that is similar to the preovulatory surge of LH in the intact, cycling ewe (Goding et al. 1969) suggests that the follicular rise in oestradiol levels may be responsible for the induction of the preovulatory gonadotrophin surge during the oestrous cycle. This concept, which is now widely accepted, has recently received confirmation by the rise in oestradiol secretion preceding a surge of LH following the induction of luteal regression with a prostaglandin analogue, 17 $\alpha$ -aryloxyprostaglandin F<sub>2 $\alpha$</sub>  (Baird & Scaramuzzi, 1976b). Secondary peaks of oestradiol secretion/

secretion have been noted on days 3-4 (Scaramuzzi et al. 1970b; Cox et al. 1971) and 8-10 (Scaramuzzi et al. 1970b) of the cycle. These peaks may well reflect the waves of follicular growth during the oestrous cycle (Smeaton & Robertson, 1971; Brand & De Jong, 1973).

The negative feedback action of oestradiol on the secretion of LH and FSH in the ewe has been described earlier in this thesis and will not be discussed further at this point.

The present investigation set out to examine the effects of active immunization against  $17\beta$ -oestradiol-6-(O-carboxymethyl)oxime-bovine serum albumin ( $E_2$ -6-BSA) on the release of gonadotrophins in the non-pregnant ewe.

Five Welsh mountain ewes were actively immunized with 1.9mg of  $E_2$ -6-BSA during the breeding season of 1974-1975 (November, 1974). Booster immunizations of 2.0 and 2.1mg of antigen were given 101 and 275 days after the primary immunization.

The incidence of oestrus after the primary immunization decreased in four of the oestradiol-immunized ewes (fig. 5.2). The fifth ewe (9R-625), however, continued to show regular oestrous cycles.

## 5.2. THE PLASMA CONCENTRATIONS OF LH AND FSH DURING ANOESTRUS IN CONTROL AND OESTRADIOL-IMMUNIZED EWES.

### 5.2.1. PROCEDURE.

In August, 1975, 14 days after the oestradiol-immunized ewes/

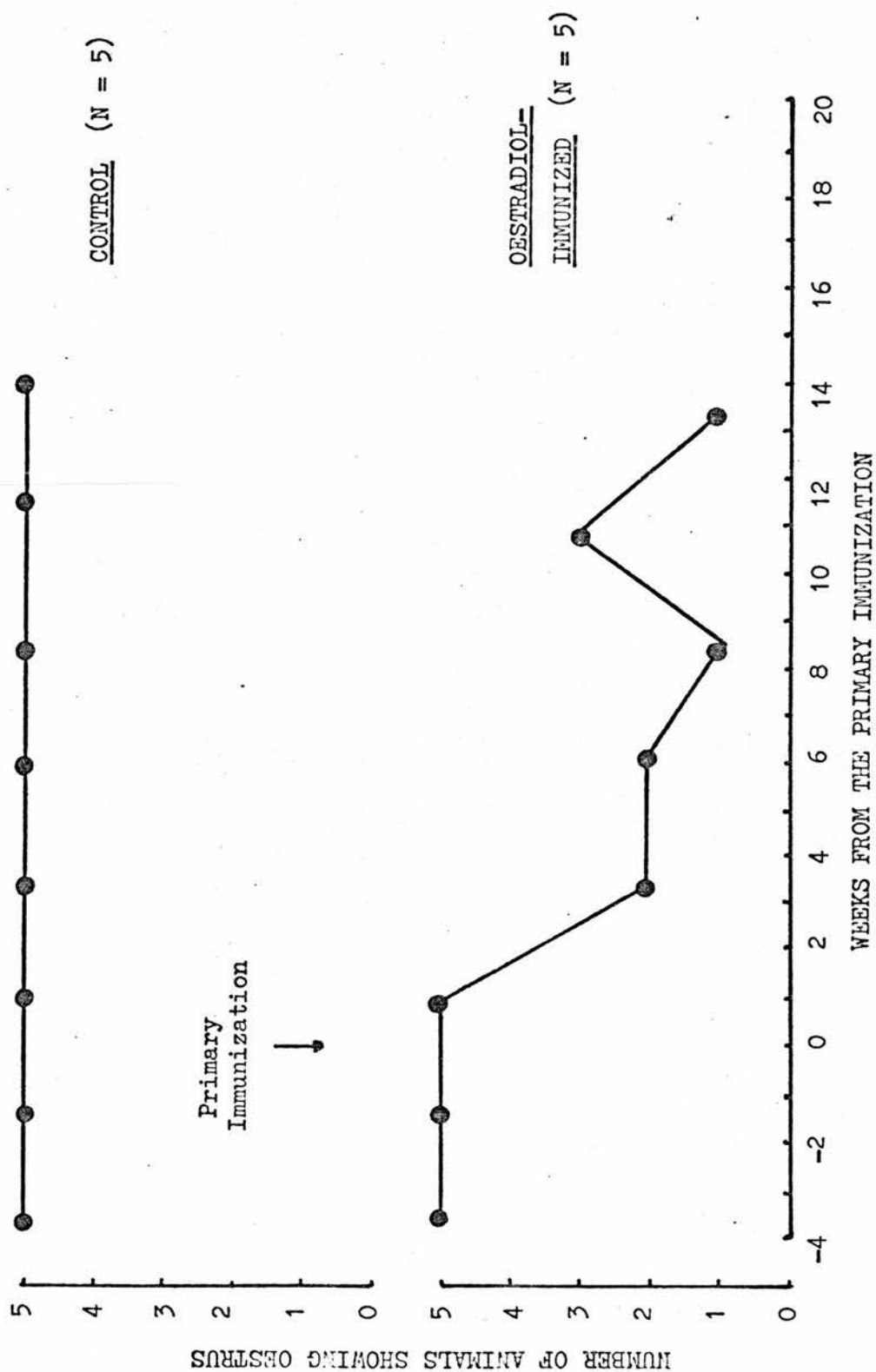


Figure 5.2. The incidence of oestrus in control and oestradiol-immunized ewes following the primary immunization

ewes had received their second booster immunization, serial blood samples (5ml) were obtained by indwelling jugular vein cannulae from the 5 oestradiol-immunized and 4 control ewes. Blood samples were taken from each animal at 15-minute intervals for 12 hours (1300-0100h).

LH was determined in all samples taken from each animal. The concentration of FSH was measured in alternate samples from each of the 5 oestradiol-immunized ewes and 3 control animals.

Plasma from each animal was pooled and the steroid antibody titre and the percentage of steroid bound after equilibrium dialysis were determined in each of these pooled samples.

#### 5.2.2. RESULTS

The steroid antibody titres, determined by charcoal absorption, in the control and oestradiol-immunized ewes are shown in Table 5.1. The binding of all steroids examined was greater in the oestradiol-immunized ewes compared to the corresponding control values. The titre of oestradiol antibodies ranged from 1:2500 to 1:17000. Significant binding of oestrone (1:100 - 1:4700) and testosterone (1:50 - 1:350) was also present. The titre of androstenedione antibodies (1:4 - 1:11), although higher than the control value, was well below the titres of the other steroid antibodies.

The mean percentages of 15-25pg of tritiated steroids bound by a 1/5 dilution of plasma from the control and oestradiol-/

TABLE 5.1.

THE TITRE<sup>a</sup> OF STEROID ANTIBODIES IN OESTRADIOL-IMMUNIZED EWES

CONTROL	<u><sup>3</sup>H-OESTRADIOL</u>	<u><sup>3</sup>H-OESTRONE</u>	<u><sup>3</sup>H-TESTOSTERONE</u>	<u><sup>3</sup>H-ANDROSTENEDIONE</u>
	N.S.	N.S.	1:1	N.S.
<hr/>				
<u>OESTRADIOL-IMMUNIZED</u>				
OR-535	1:15000	1:4100	1:80	1:7
9R-727	1:7200	1:2100	1:70	1:11
9R-625	1:2500	1:100	1:100	1:4
OR-606	1:2900	1:200	1:50	1:9
OR-872	1:17000	1:4700	1:350	1:6

<sup>a</sup>Titre = The initial dilution of plasma which bound 50% of 15-20pg of tritiated steroid.

N.S. = &lt;50% binding of the tritiated steroid at a dilution of 1:1.

oestradiol-immunized ewes are shown in Table 5.2. The binding of all steroids examined was significantly higher in the oestradiol-immunized animals.

The concentrations of LH during anoestrus in jugular venous plasma from the four control ewes are shown in figure 5.3. The concentration of LH was low (mean basal levels: 0.23-0.64 ng/ml) apart from one spontaneous peak of LH which occurred in each of the control ewes with a peak height ranging from 2.67 to 5.35 ng/ml. In three ewes (8R-542, 8R-676, 8R-602; fig. 5.3), the peak of LH occurred at about 2100h. In the fourth animal (8R-621; fig. 5.3), the LH peak occurred at 1630h, some 4½ hours earlier than in the other animals.

The concentrations of FSH, determined in alternate samples from each of 3 control ewes, are also included in figure 5.3. The mean concentration of FSH varied considerably between the animals, ranging from 27.5 to 60.0 ng/ml. However, little variation in the concentration of FSH was apparent during the 12-hour period within each animal, although an elevation in the concentration of FSH occurred at the same time, or just before, the time of the LH peak (fig. 5.3).

The patterns of LH and FSH release during anoestrus in the five oestradiol-immunized ewes are illustrated in figures 5.4 and 5.5. The pulsatile release of LH was apparent in all the oestradiol-immunized ewes with 5 or 6 peaks in the concentration of LH occurring in each animal during the 12-hour period. The mean peak height and the mean basal level of LH were significantly higher in the oestradiol-immunized animals compared to the corresponding control values (figure 5.6). A wide variation/

TABLE 5.2.

THE PERCENTAGE ( $M \pm S.E.$ ) OF 15-25pg OF TRITIATED STEROID BOUND BY A 1/5 DILUTION OF PLASMA  
FROM THE CONTROL AND OESTRADIOL-IMMUNIZED EWES DETERMINED BY EQUILIBRIUM DIALYSIS

	$^3$ H-OESTRADIOL	$^3$ H-OESTRONE	$^3$ H-TESTOSTERONE	$^3$ H-ANDROSTENEDIONE
CONTROL	88.4 $\pm$ 1.5 (n=4)	80.1 $\pm$ 3.1 (n=4)	81.7 $\pm$ 1.1 (n=4)	57.2 $\pm$ 2.2 (n=4)
OESTRADIOL- IMMUNIZED	99.5 $\pm$ 0.4* (n=5)	99.7 $\pm$ 0.1* (n=5)	98.8 $\pm$ 0.5* (n=5)	92.7 $\pm$ 2.3* (n=5)

\*  $p < 0.001$ . Immunized vs. Control (Student's t-test).



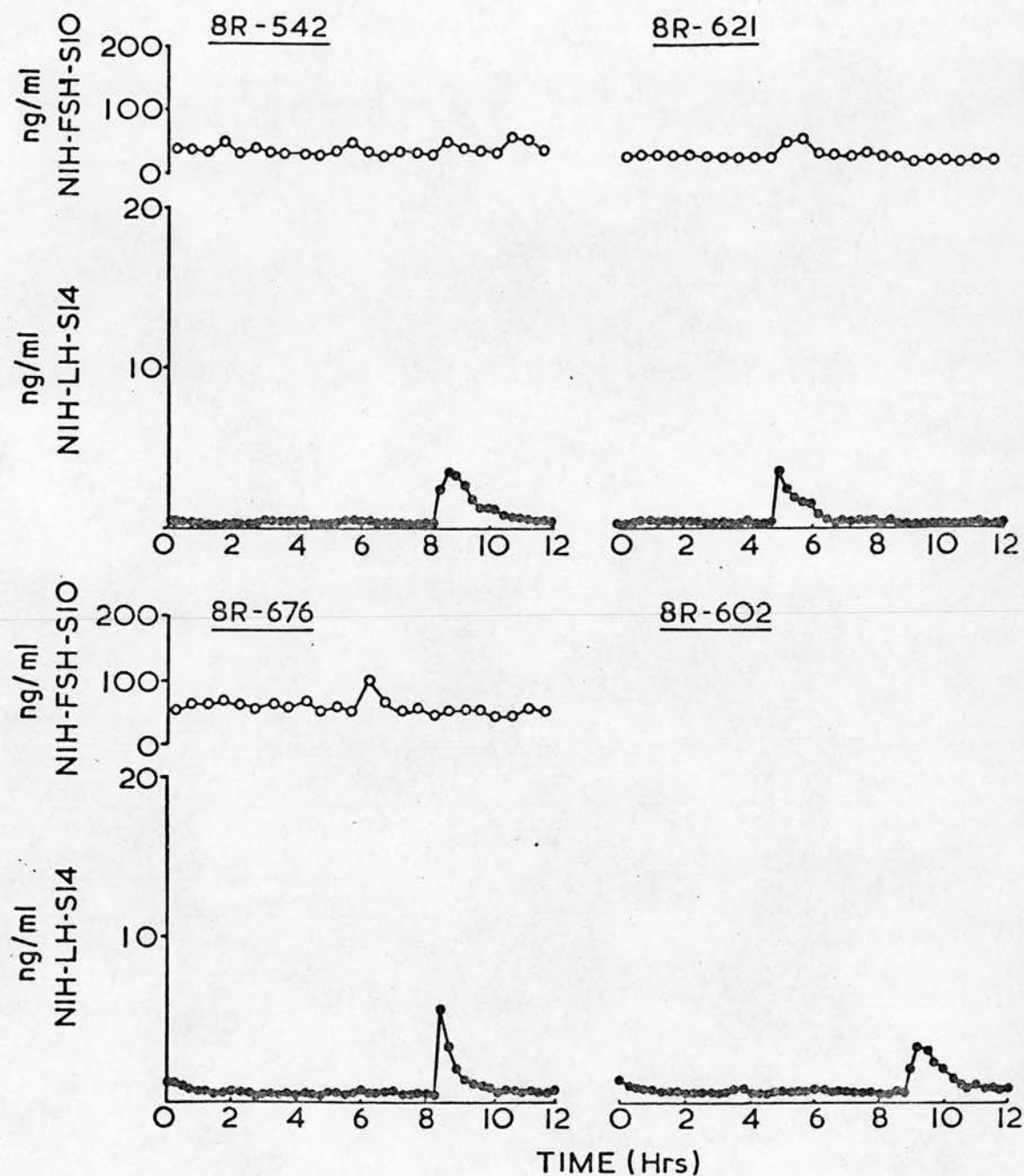
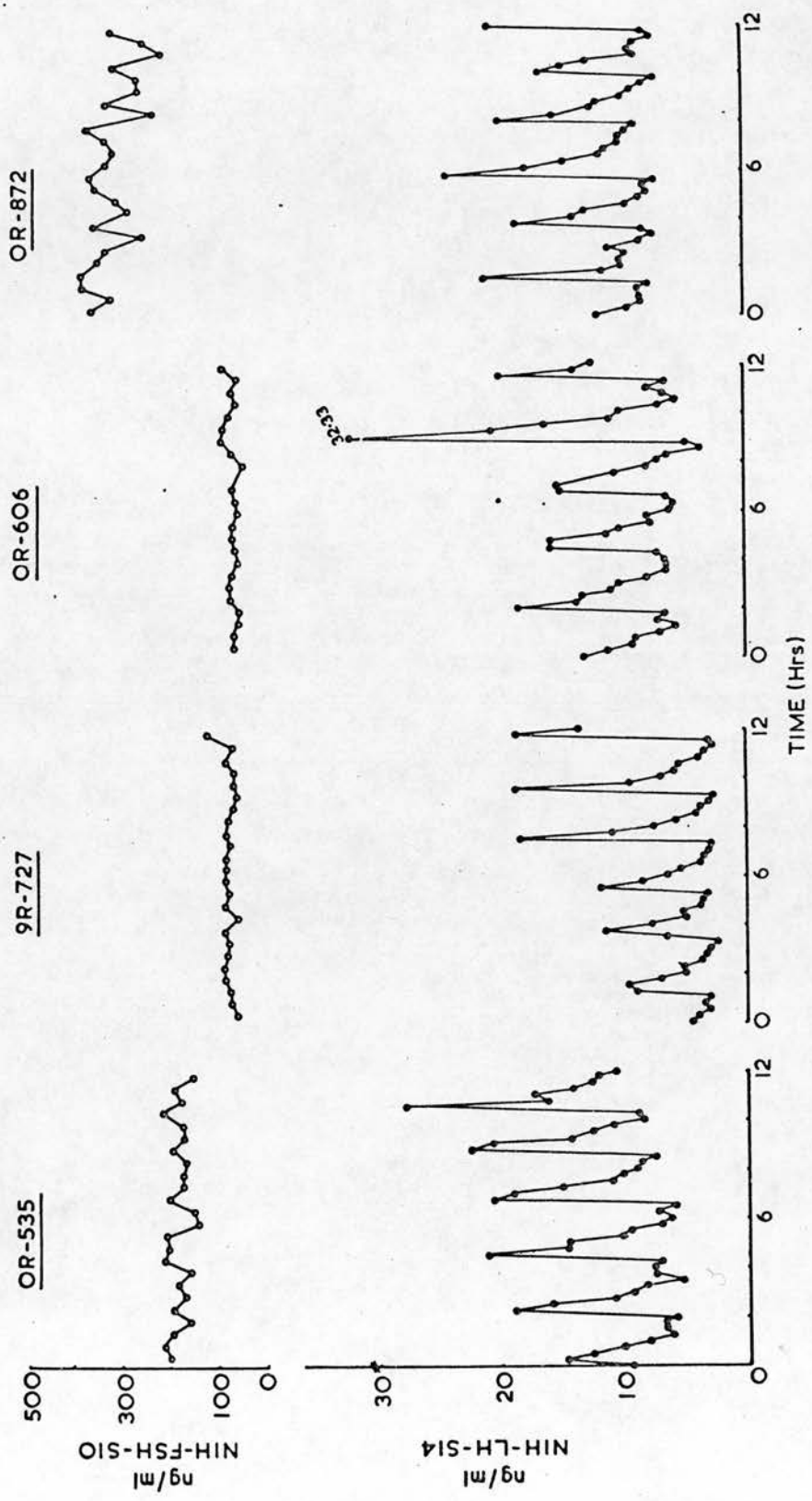


Figure 5.3. The plasma concentrations of LH and FSH during anoestrus in control ewes.

Samples were taken at 15-minute intervals for 12 hours. LH was assayed in every sample. FSH was measured in alternate samples from 3 control ewes.



**Figure 5.4.** The plasma concentrations of LH and FSH in four oestradiol-immunized ewes during anoestrus. Samples were taken at 15-minute intervals for 12 hours. LH was assayed in every sample. FSH was measured in alternate samples.

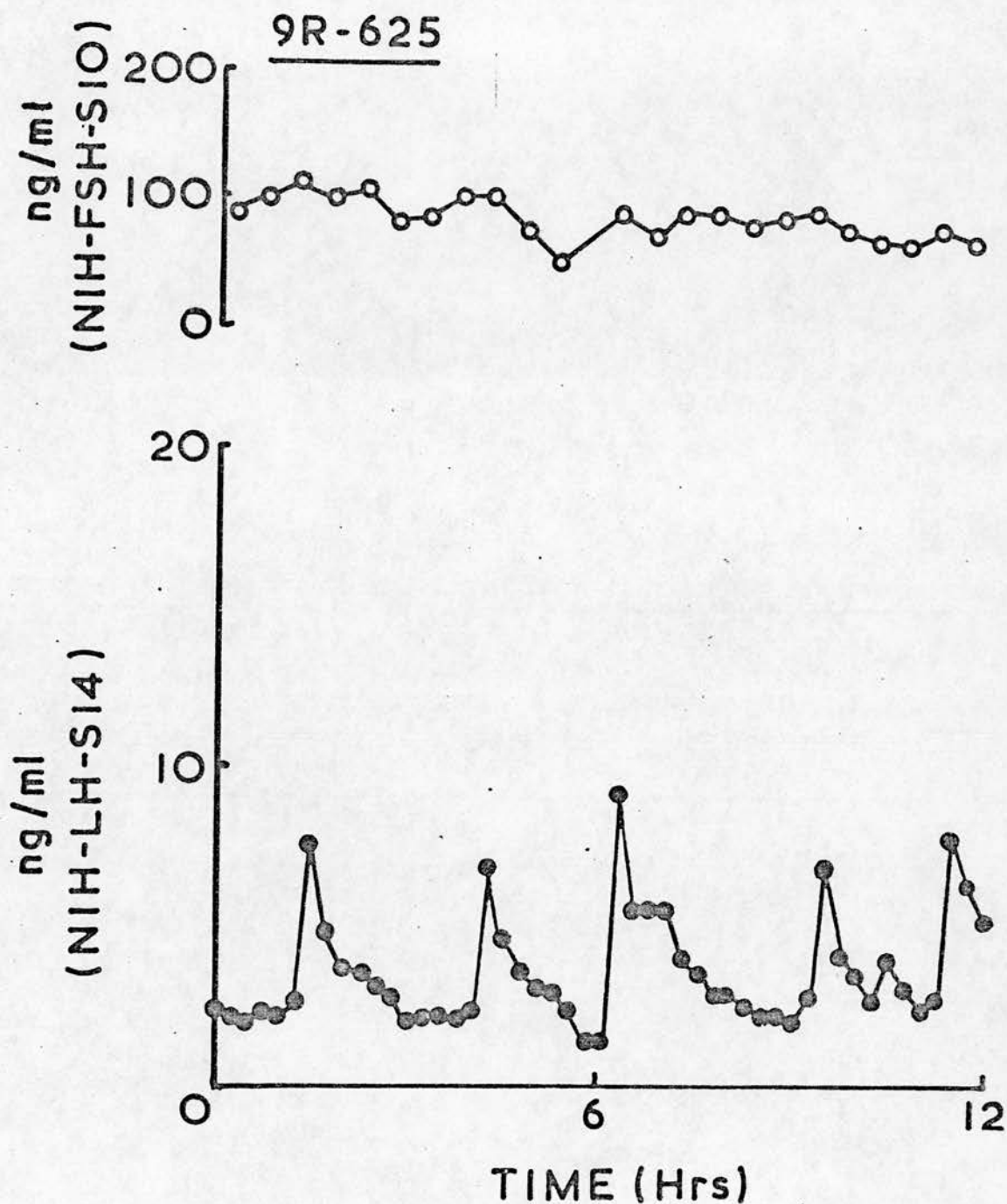


Figure 5.5. The plasma concentrations of LH and FSH during anoestrus in an oestradiol-immunized ewe.

Samples were taken at 15-minute intervals for 12 hours.

LH was assayed in every sample. FSH was measured in alternate samples.

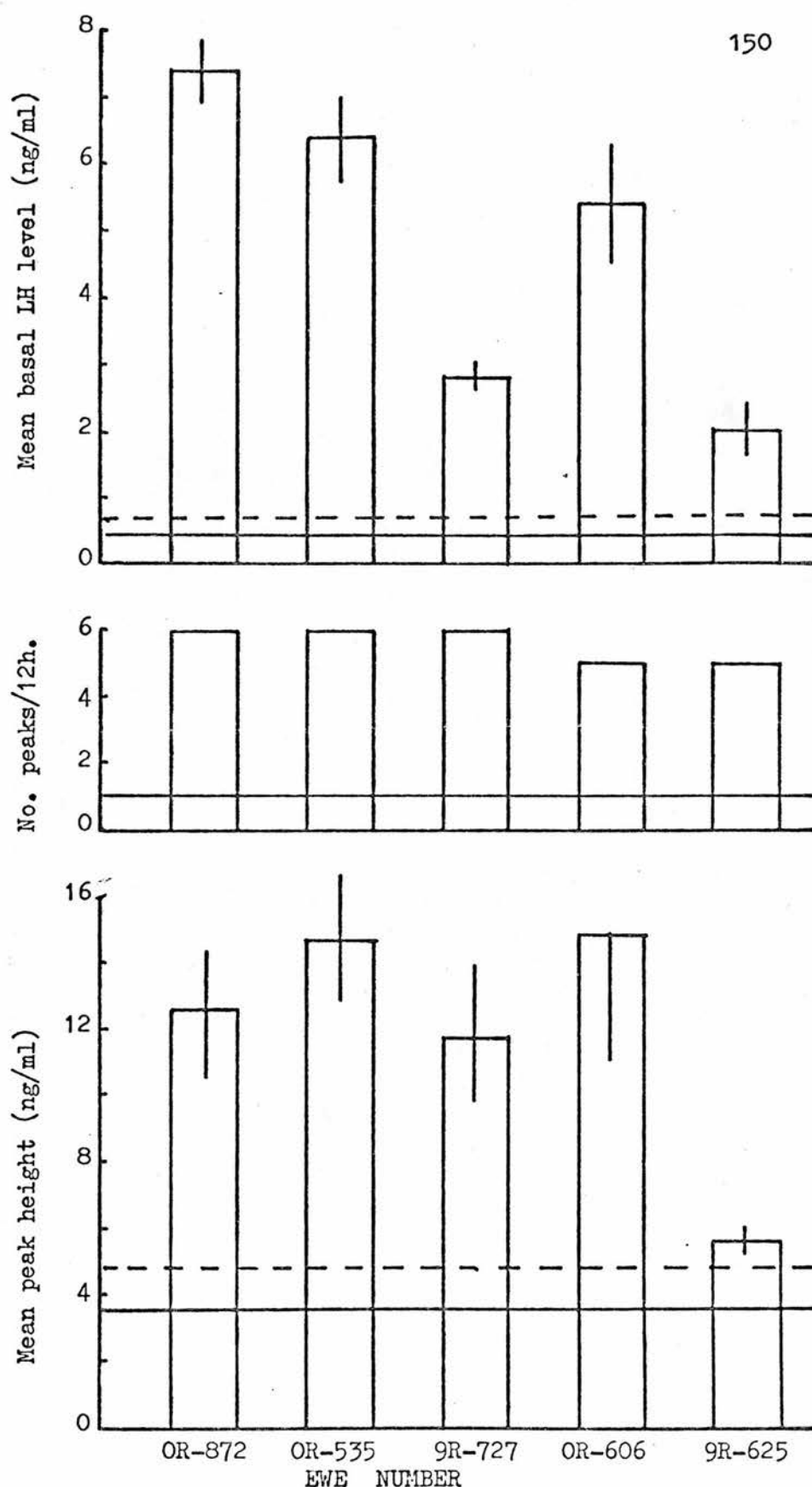


FIGURE 5.6. The pattern of LH release during anoestrus in oestradiol-immunized ewes.

The values are arranged in order of decreasing oestradiol antibody titre.

The vertical bar represents the range (mean  $\pm$  S.E.). The horizontal line represents the mean, and the broken line, the range (mean  $\pm$  2 S.D.) of the control group.

wide variation in the concentration of LH was apparent between the oestradiol-immunized ewes. The two ewes with the highest oestradiol antibody titres showed the greatest elevation in the concentration of LH (OR-872, OR-535; fig. 5.4). The level of LH in the remaining 3 oestradiol-immunized ewes, however, was not directly related to the oestradiol antibody titre since the animal with a high antibody titre (9R-727; 1:7200) showed a lower level of LH than a ewe (OR-606; fig. 5.4.) with a lower antibody titre (1:2900). The fifth ewe (9R-625), which continued to show regular oestrous cycles after the primary immunization and had the lowest titre of oestradiol antibodies (1:2500), also showed the lowest level of LH (fig. 5.5).

The concentration of FSH was greatly increased in the two ewes with the highest oestradiol antibody titres (OR-872; OR-535; fig. 5.4) compared to the control animals. In the remaining oestradiol-immunized ewes, however, the mean concentration of FSH was not significantly different from the control value (fig. 5.7). Although fluctuations in the concentration of FSH occurred during the 12-hour period in the oestradiol-immunized ewes (figs. 5.4 and 5.5), no clear relationship between the release of LH and FSH could be ascertained.

### 5.2.3. DISCUSSION

All five oestradiol-immunized ewes responded to the immunization procedure as demonstrated by the presence of significant/

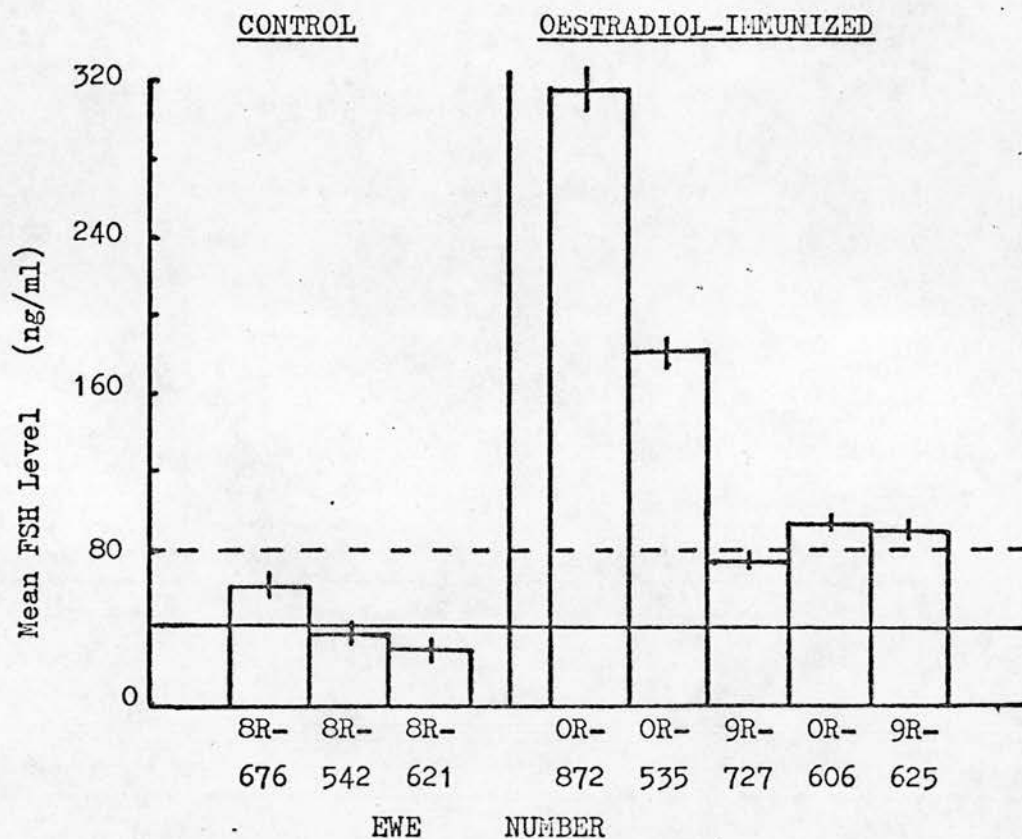


Figure 5.7 The mean concentration of FSH during anoestrus in control and oestradiol-immunized ewes.

The values of the oestradiol-immunized ewes are arranged in order of decreasing oestradiol antibody titre.

The vertical bars represent the range (mean  $\pm$  S.E.).

The horizontal line represents the mean, and the broken line the range (mean + 2 S.D.) of the control group.



significant titres of oestradiol antibodies. The finding that the binding of oestrone in the oestradiol-immunized animals was also increased may have been due to the presence of antibodies directed towards the phenolic A-ring which is common to both the  $E_2$ -6-BSA and oestrone molecules. Similarly, the C-17 hydroxyl group, common to both  $E_2$ -6-BSA and testosterone, may have contributed towards the significant testosterone antibody titres in the oestradiol-immunized animals. Conversely, the absence of a functional group common to both the  $E_2$ -6-BSA and androstenedione molecules may have resulted in the absence of antibodies capable of binding androstenedione.

The elevation in the plasma concentration of LH in the oestradiol-immunized ewes confirmed a similar elevation of LH in female rhesus monkeys (Ferin et al. 1974) and rats (Hillier et al. 1975a,b) actively immunized against oestradiol-protein conjugates.

The negative feedback action of oestradiol in the control of LH secretion in the ewe has been widely accepted. The present findings are consistent with the hypothesis that the presence of oestradiol antibodies had reduced the level of circulating biologically active oestradiol thereby reducing the negative feedback control of LH secretion in the ewe.

It is interesting to compare the pattern of LH release in the oestradiol-immunized ewes with the levels of LH in the ovariectomized ewes described previously (chapter 4) (Table 5.3). The pulsatile pattern of LH release, characteristic of ovariectomized ewes, was also apparent in the oestradiol-immunized animals, although the frequency of spontaneous LH peaks/

TABLE 5.3

## THE PATTERN OF LH AND FSH RELEASE DURING ANOESTRUS IN OESTRADIOL-IMMUNIZED EWES AND

## OVARIECTOMIZED-HYSTERECTOMIZED ANIMALS

	OESTRADIOL-IMMUNIZED					OVARIECTOMIZED-HYSTERECTOMIZED				
	OR-872	OR-535	OR-606	9R-727	9R-625	6R-125	6R-121	7R-227	7R-522	7R-465
<u>LH.</u>										
mean basal level <sup>a</sup> (ng/ml) ( $\pm$ SD) (n=5)	7.37 $\pm$ 0.50	6.29 $\pm$ 0.74	5.42 $\pm$ 0.91	2.74 $\pm$ 0.20	2.01 $\pm$ 0.35	3.62 $\pm$ 1.12	7.64 $\pm$ 1.40	10.20 $\pm$ 1.33	4.94 $\pm$ 0.86	4.74 $\pm$ 0.67
Number of LH peaks/12 hours	6	6	6	5	5	10	9	10	13	10
mean ( $\pm$ SE) peak height (ng/ml)	12.47 $\pm$ 1.02	14.60 $\pm$ 1.77	14.78 $\pm$ 3.15	11.94 $\pm$ 1.95	5.56 $\pm$ 0.43	30.86 $\pm$ 5.50	24.51 $\pm$ 0.27	14.64 $\pm$ 1.44	9.84 $\pm$ 0.58	31.03 $\pm$ 3.64
<u>FSH.</u>										
mean concentration (ng/ml) ( $\pm$ SE) (n = 24)	317.8 $\pm$ 9.6	181.5 $\pm$ 4.4	89.7 $\pm$ 2.2	75.6 $\pm$ 2.6	85.2 $\pm$ 3.2	400.0 $\pm$ 6.4	-	356.9 $\pm$ 8.2	-	157.0 $\pm$ 4.7

n = number of determinations.

<sup>a</sup> mean basal level = the mean of the 5 lowest concentrations of LH measured during the 12-hour period.



peaks was lower in the oestradiol-immunized animals (Table 5.3). The basal levels of LH in the oestradiol-immunized ewes were similar to those found in the ovariectomized animals (Table 5.3).

The elevation in the concentration of FSH in the two ewes with the highest oestradiol antibody titres was consistent with the elevated levels of FSH found in the ovariectomized group (Table 5.3). In contrast, the level of FSH in the three ewes with the lower oestradiol antibody titres was not significantly altered compared to the control value. It is tempting to suggest that in the ewe, the release of FSH is more sensitive to the negative feedback action of oestradiol than the release of LH, as has been suggested in the human (Tsai & Yen, 1971; Kulin & Reiter, 1972). Therefore, the incomplete neutralization of oestradiol, suggested by the relatively low oestradiol antibody titres, may have resulted in a sufficient amount of biologically active oestradiol remaining to exert a negative feedback action on the more sensitive control of FSH secretion, whilst reducing the negative feedback action on the less sensitive control of LH secretion.

Another alternative may lie in the hypothesis that androgens may in some way enhance the release of FSH as suggested by the presence of lowered plasma FSH levels in the ewes immunized against androstenedione (chapter 4). In this case, the presence of testosterone antibodies in the oestradiol-immunized ewes may have reduced the level of biologically active androgen thereby reducing the release of FSH. This factor, counteracting the reduced negative feedback/

feedback action of oestradiol may have given rise to the relatively normal FSH levels seen in 3 oestradiol-immunized ewes.

The similarity of the patterns of LH release in the oestradiol-immunized and ovariectomized ewes suggests that oestradiol is the major ovarian steroid responsible for the suppression of LH secretion in the ewe.

### 5.3. THE PLASMA CONCENTRATIONS OF LH AND FSH DURING THE BREEDING SEASON IN CONTROL AND OESTRADIOL-IMMUNIZED EWES.

#### 5.3.1. PROCEDURE

During September 1975, progesterone sponges (Synchromate: G.D. Searle & Co.) containing 20mg of fluorogestone acetate (S.C. 9880: 17-acetoxy-9-fluoro-11 $\beta$ -hydroxypregnen-4-ene-3:20-dione) were inserted into the vaginae of the nine control and oestradiol-immunized ewes. The sponges were removed 12 days later and the ewes were run with a vasectomized ram with a marking harness for the detection of oestrus. All the control ewes showed oestrus 3-4 days after the withdrawal of the progesterone sponges. None of the oestradiol-immunized ewes came into heat during this period. However, one oestradiol-immunized ewe (9R-625) showed oestrus 31 days after the removal of the progesterone sponges. The remaining ewes were in a state of anoestrus throughout the experimental period.

The control ewes underwent a complete oestrous cycle before the experiment was performed, 82 days after the oestradiol/

oestradiol-immunized ewes had received their second booster immunization.

Serial samples (5ml) of jugular venous blood were taken from the control ewes at 15-minute intervals for 6 hours on each of four days during the oestrous cycle (Table 5.4).

The four oestradiol-immunized ewes which failed to show behavioural oestrus were sampled at 15-minute intervals for 6 hours on day "10" of the cycle had they shown heat at the same time as the control animals. The fifth ewe (9R-625) was sampled at 15-minute intervals for 6 hours two days before the first oestrus and on each of 4 days during the subsequent cycles (Table 5.4). Where appropriate, the results were normalized around the day of the second oestrus (day 0).

The concentration of LH was measured in every sample taken from all animals. The level of FSH was determined in alternate samples taken from each of the 5 oestradiol-immunized ewes and 3 control animals.

The concentration of progesterone was measured in the first sample taken from each animal at each sampling period.

Steroid antibody titres and the percentage of steroid bound after equilibrium dialysis were determined after pooling the samples taken from each animal.

### 5.3.2. RESULTS.

The titres of steroid antibodies in the control and oestradiol-immunized ewes are shown in Table 5.5. Since the experiment was performed 82 days after a booster immunization, the antibody titres were lower in the oestradiol-immunized ewes than those found during the earlier experiment.

The/

TABLE 5.4.  
THE SAMPLING PROTOCOL DURING THE BREEDING SEASON

	<u>Length of the cycle</u> (days)	<u>Sampling days<sup>a</sup></u>
<u>CONTROL</u>		
8R-602	17	-9   -5   -1   +1
8R-621	17	-9   -5   -1   +1
8R-676	16	-10   -6   -2   +1
8R-542	15	-7   -3   +1
<u>OESTRADIOL- IMMUNIZED</u>		
9R-625	20	A   -13   -8   -3   +2
OR-872	NBO	"10"
OR-606	NBO	"10"
OR-535	NBO	"10"
9R-727	NBO	"10"

<sup>a</sup>sampling days = days from the second oestrus (day 0). Blood samples were taken at 15-minute intervals for 6-hours on each sampling day.

NBO = no behavioural oestrus. Animals were sampled on the expected day "10" had they shown oestrus at the same time as the controls.

A = animal also sampled two days before 1st oestrus.

TABLE 5.5.  
THE TITRE<sup>a</sup> OF STEROID ANTIBODIES IN OESTRADIOL-IMMUNIZED EWES

CONTROL	<sup>3</sup> H-OESTRADIOL	<sup>3</sup> H-OESTRONE	<sup>3</sup> H-TESTOSTERONE	<sup>3</sup> H-ANDROSTENEDIONE
	N.S.	N.S.	1:1	N.S.
<hr/>				
OESTRADIOL- IMMUNIZED				
OR-535	1:6400	1:2600	1:30	1:1
9K-727	1:4000	1:2200	1:100	1:5
9R-625	1:1300	1:250	1:220	1:2
OR-606	1:1400	1:250	1:37	1:1
OR-872	1:7200	1:2600	1:64	1:9

<sup>a</sup>Titre = The initial dilution of plasma which bound 50% of 15-20pg of tritiated steroid.

N.S. = <50% binding of the tritiated steroid at a dilution of 1:1.

The titre of oestradiol antibodies ranged from 1:1300 to 1:7200. Significant titres of oestrone (1:250 - 1:2600) and testosterone (1:30 - 1:220) antibodies were present. Negligible binding of androstenedione, compared to the other steroid antibody titres, was found in the oestradiol-immunized animals.

The binding of all steroids, determined by equilibrium dialysis was significantly higher in the oestradiol-immunized ewes (Table 5.6).

The concentration of progesterone during the oestrous cycle in the control ewes is shown in figure 5.8. It is apparent that maximal levels of progesterone were present in peripheral plasma from days -10 to -3 of the cycle. The level of progesterone was low (0.1 and 0.2 ng/ml) in the two ewes sampled on the day before oestrus.

The concentrations of LH during the oestrous cycle in the four control ewes are shown in figures 5.9 and 5.10. The basal level of LH was low (range 0.32-1.23 ng/ml) during the mid- and late-luteal phase of the cycle (days -10 to -3) when the concentration of progesterone was high. In the two control animals sampled on the day before oestrus, when the level of progesterone had fallen, the level of LH was higher than the levels of LH seen during the luteal phase of the cycle (fig. 5.9).

The plasma concentrations of FSH during the oestrus cycle in 3 control ewes are also included in figures 5.9 and 5.10. A wide variation in the level of FSH was apparent between individual animals (range 32.7-127.0 ng/ml). The concentration of FSH, however, was relatively constant throughout the cycle within each ewe although the level of FSH/

**TABLE 5.6.**  
**THE PERCENTAGE (M<sup>±</sup>S.E.) OF 15-25pg OF TRITIATED STEROID BOUND BY A 1/5 DILUTION OF PLASMA**  
**FROM THE CONTROL AND OESTRADIOL-IMMUNIZED EWES DETERMINED BY EQUILIBRIUM DIALYSIS**

	<u><sup>3</sup>H-OESTRADIOL</u>	<u><sup>3</sup>H-OESTRONE</u>	<u><sup>3</sup>H-TESTOSTERONE</u>	<u><sup>3</sup>H-ANDROSTENEDIONE</u>
<u>CONTROL</u>	84.9±0.1 (n=4)	78.0±0.9 (n=4)	83.2±0.8 (n=4)	53.4±2.1 (n=4)
<u>OESTRADIOL- IMMUNIZED</u>	99.6±0.1* (n=5)	99.6±0.1* (n=5)	98.8±0.4* (n=5)	90.1±3.0* (n=5)

\*  $p < 0.001$ . Immunized vs. Control (Student's t-test).



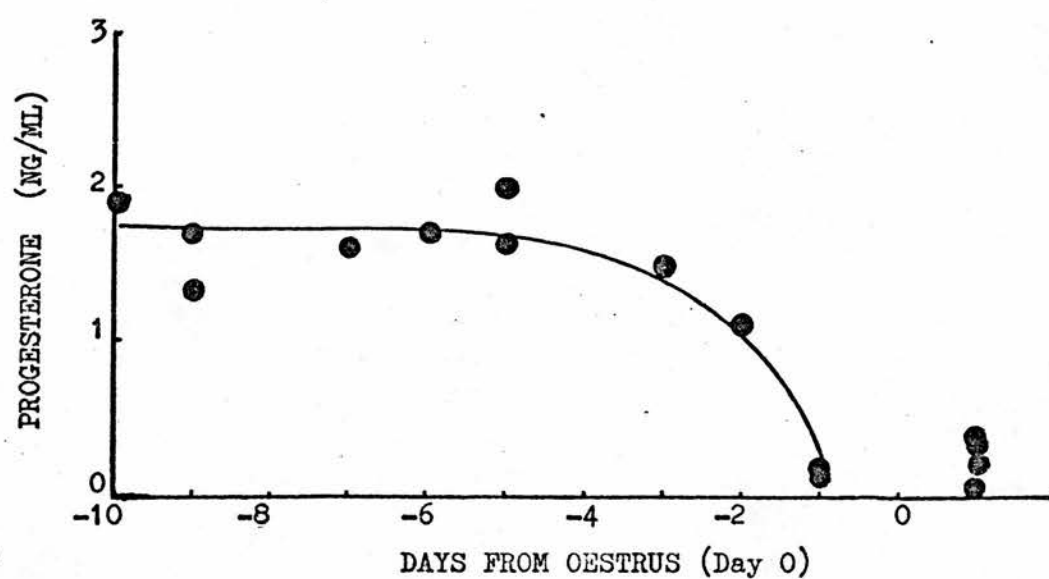


Figure 5.8.  
The concentration of progesterone during the oestrus cycle  
in control ewes.

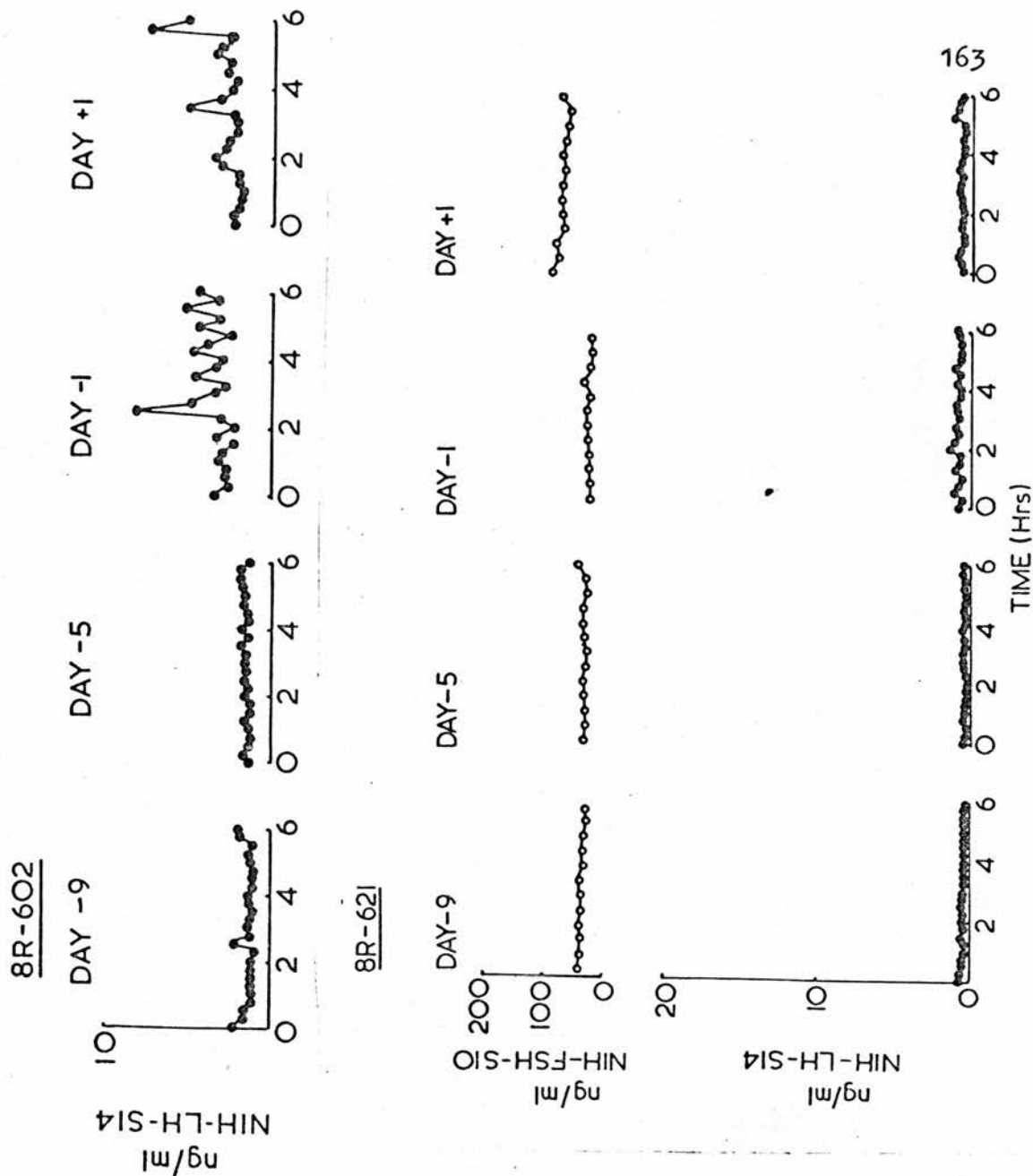
**Figure 5.9.**

The plasma concentrations of LH and FSH during the oestrous cycle in control ewes.

Samples were taken at 15-minute intervals for 6 hours on each of 4 days during the oestrous cycle.

LH was assayed in every sample.

FSH was measured in alternate samples.



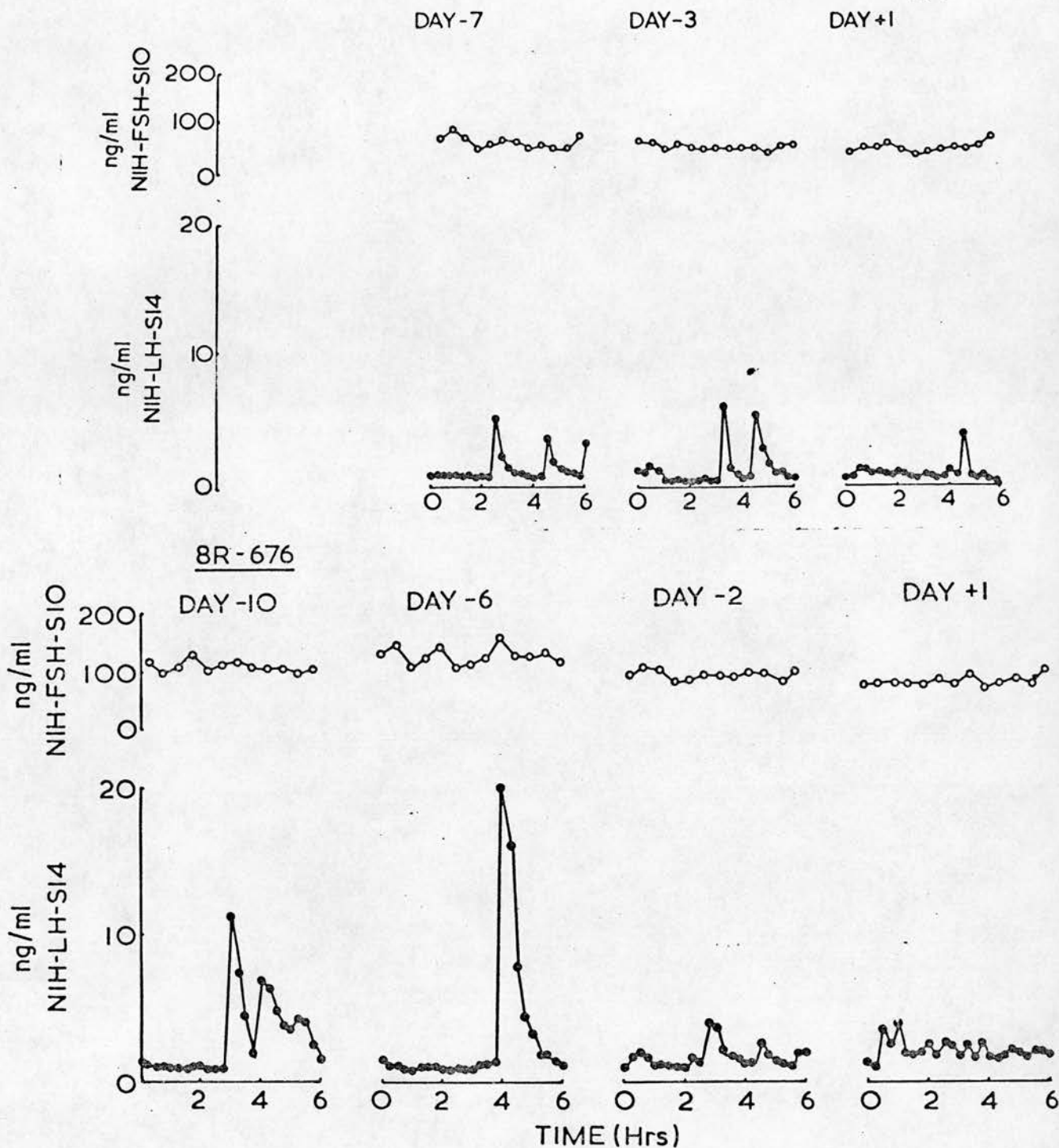


Figure 5.10 The plasma concentrations of LH and FSH during the oestrous cycle in two control ewes.

Samples were taken at 15-minute intervals for 6 hours at each sampling period.

LH was assayed in every sample. FSH was measured in alternate samples.

FSH tended to be lower during the follicular phase in two ewes (fig. 5.9: 8R-621, day -1; fig. 5.10: 8R-676, day -2) when the level of progesterone was falling (fig. 5.8).

The plasma concentrations of LH and FSH in the 4 oestradiol-immunized ewes which failed to show behavioural oestrus are shown in figure 5.11. A regular pulsatile release of LH was apparent in the two ewes with the highest oestradiol antibody titres (fig. 5.11: OR-872, OR-535) with 3 or 1 peaks of LH occurring during the 6 hour period. The mean basal level of LH in all the oestradiol-immunized ewes was greater than the highest basal level of LH found during the oestrous cycle in the control ewes (fig. 5.12).

Fluctuations in the concentration of FSH occurred during the 6 hour period within each oestradiol-immunized ewe. However, no clear relationship between the release of LH and FSH was apparent (fig. 5.11). The mean concentration of FSH varied considerably between the oestradiol-immunized ewes and was significantly higher in the two ewes with highest oestradiol antibody titres (fig. 5.13). The mean level of FSH in the other two anoestrous oestradiol-immunized ewes, although greater than the mean concentrations of FSH during the oestrous cycle in individual control ewes, was not significantly different from the mean of the control group due to the large variation within this group.

The plasma concentrations of LH and FSH during the oestrous cycle in the fifth oestradiol-immunized ewe are shown in figure 5.14. The concentration of LH was higher than those levels seen during the oestrous cycle in the control animals. However, the inverse relationship between the concentration of /

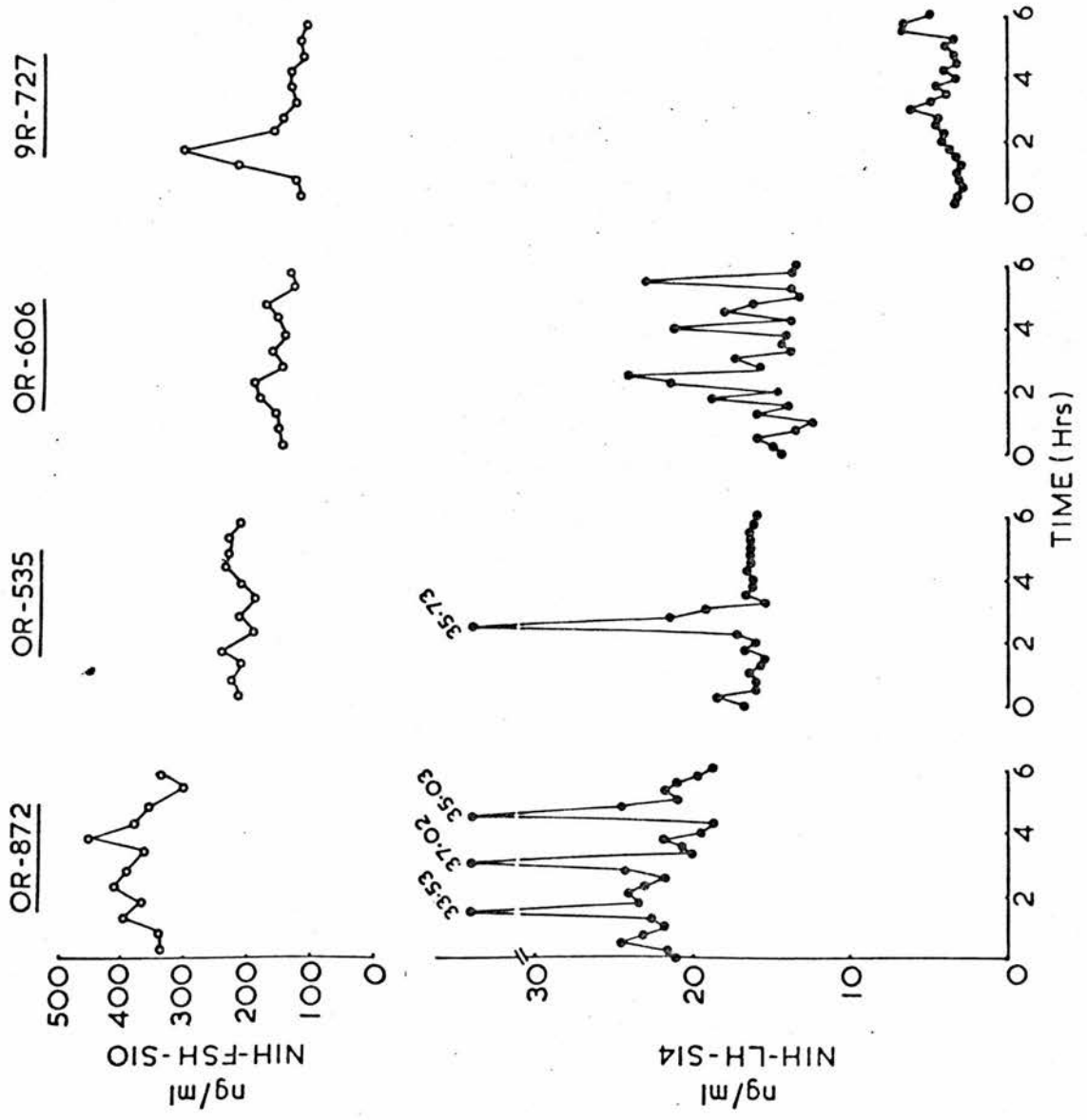


Figure 5.11.  
The plasma concentrations of LH and  
FSH during the breeding season in  
four oestrous oestradiol-immunized  
ewes.  
 Samples were taken at 15-minute  
 intervals for 6 hours.  
 LH was assayed in every sample.  
 FSH was measured in alternate samples.

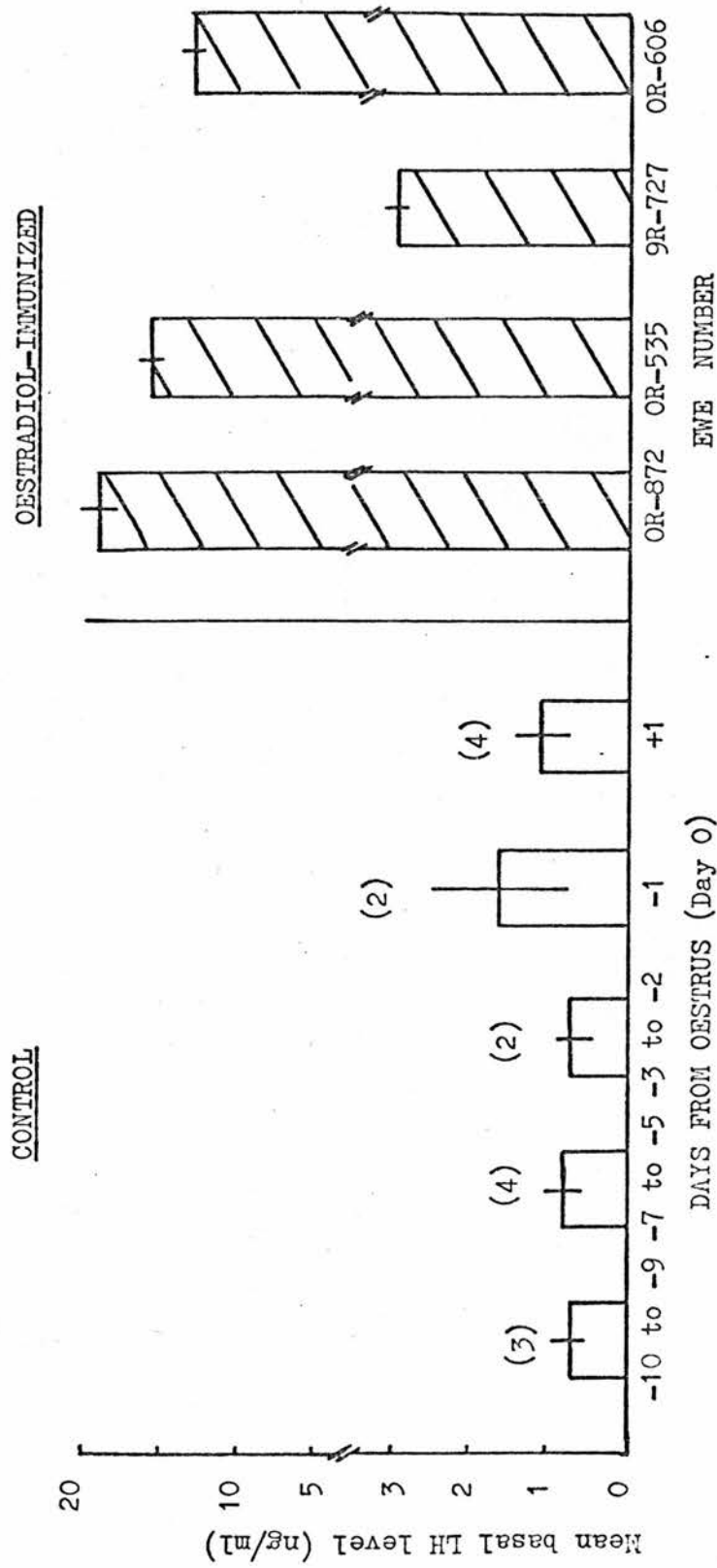
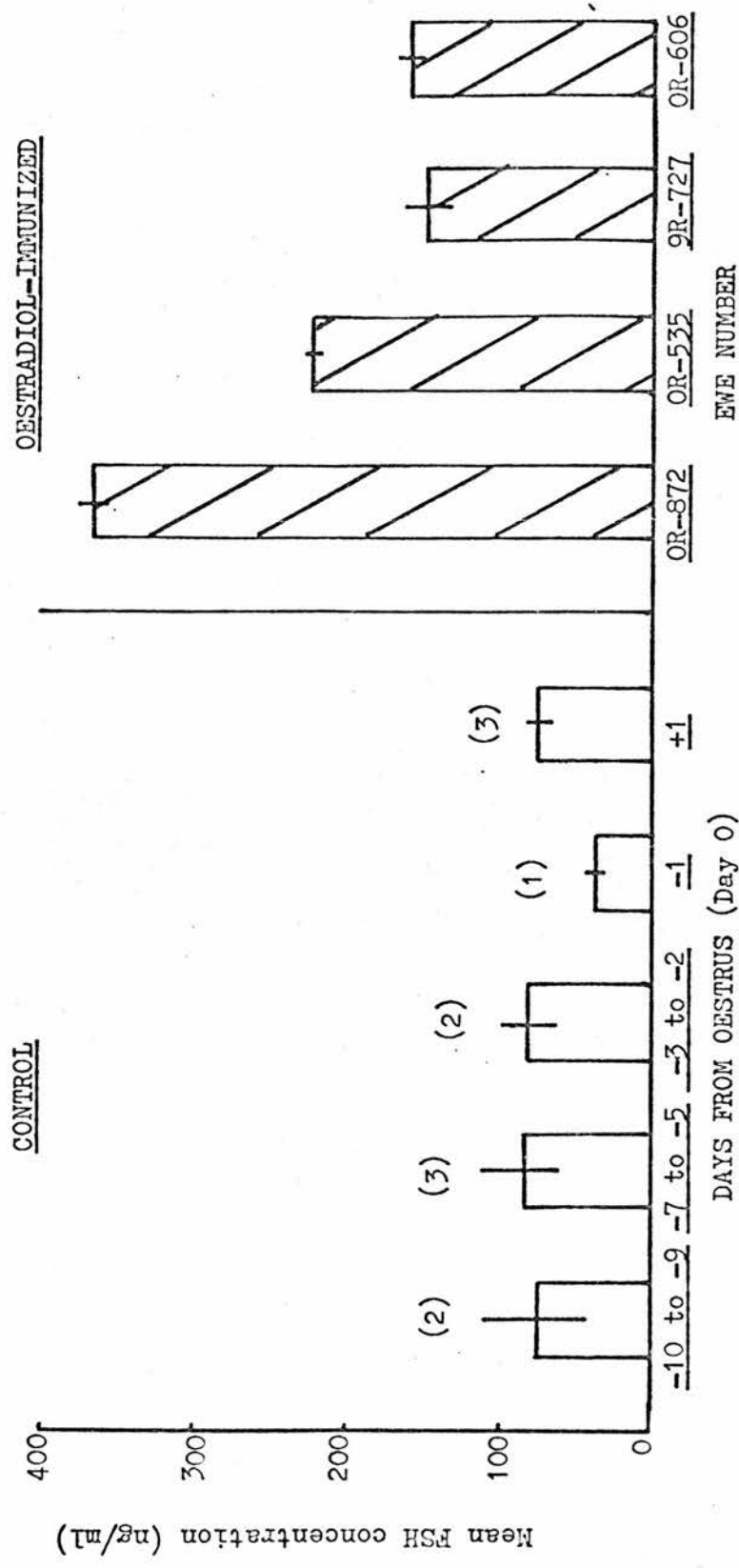


Figure 5.12. The mean basal level of LH in control and anoestrous oestradiol-immunized ewes during the breeding season.

The oestradiol-immunized ewes are arranged in order of decreasing oestradiol antibody titre.  
 The number in parentheses represents the number of control animals.  
 The vertical line represents the range (mean  $\pm$  S.D.).



**Figure 5.13.** The mean concentration of FSH during the breeding season in control and anoestrous oestradiol-immunized ewes.

The oestradiol-immunized ewes are arranged in order of decreasing oestradiol antibody titre. The number in parentheses represents the number of control animals. The vertical line represents the range (mean ± S.E.).



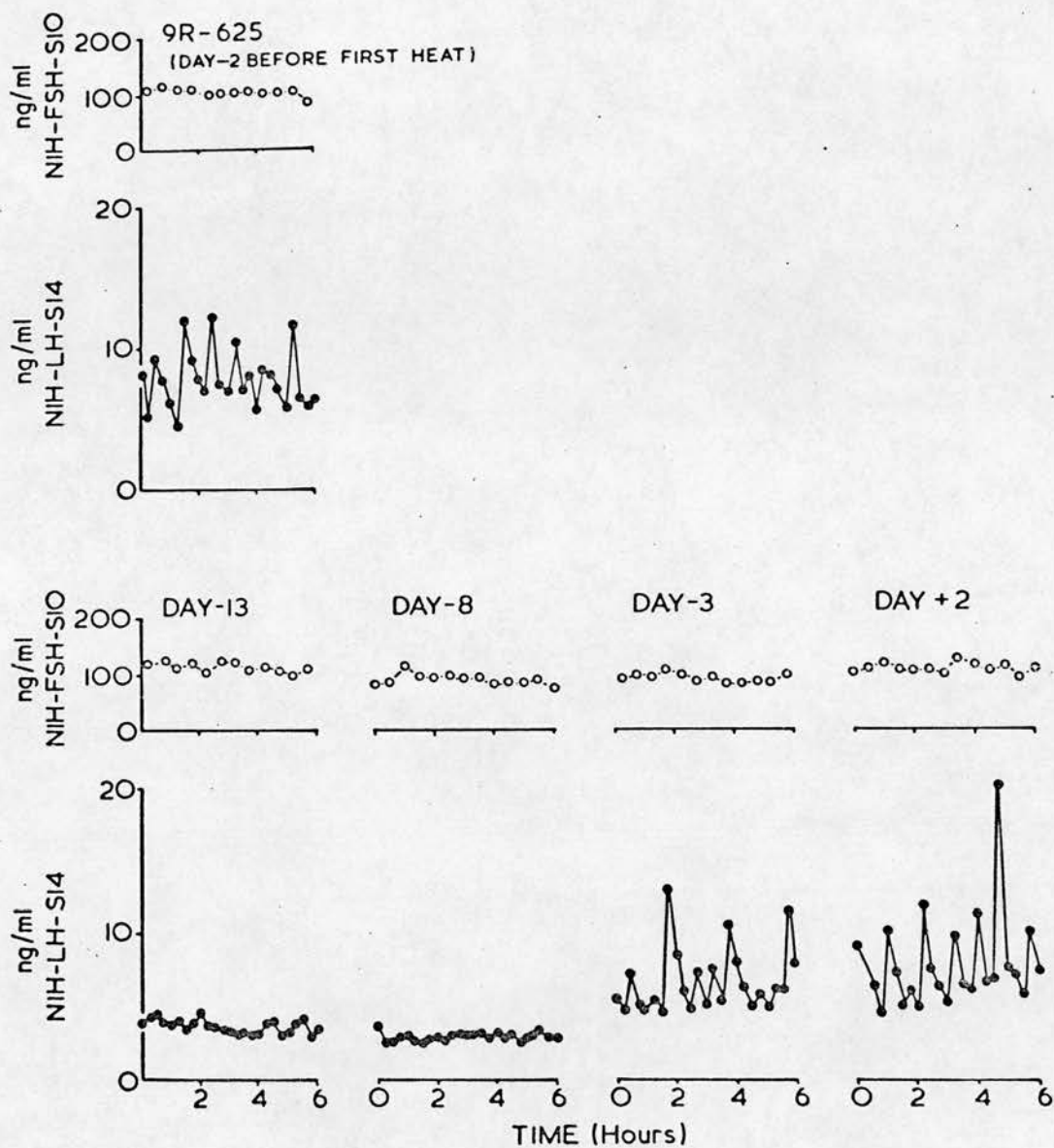


Figure 5.14. The plasma concentrations of LH and FSH during the oestrous cycle in an oestradiol-immunized ewe.

Samples were taken at 15-minute intervals for 6 hours at each of the sampling periods. LH was assayed in every sample. FSH was measured in alternate samples.

of progesterone and the basal level of LH (fig. 5.15) was still apparent since the concentration of LH was higher on days -3 and +2 (fig. 5.14) when the progesterone level was low (0.1 and 0.6 ng/ml). The level of FSH in this animal was within the control range throughout the oestrous cycle.

### 5.3.3. DISCUSSION.

The concentrations of LH, FSH and progesterone during the oestrous cycle in the control animals were similar to those described in the previous chapter (chapter 4). The level of progesterone remained at maximal levels between days -10 and -3 after which the level fell to reach a low value on the day before oestrus. A similar cyclical pattern in the concentration of progesterone in peripheral plasma during the oestrous cycle of the ewe has been reported previously (Thorburn et al. 1969; McNatty et al. 1973; Baird et al. 1976a). Low levels of LH were found during the luteal phase of the cycle with a rise in the concentration of LH during the follicular phase when the level of progesterone had fallen. Little variation in the level of FSH was apparent during the oestrous cycle within individual animals. A peak of FSH, reported to occur on the day following oestrus (L'Hermite et al. 1972; Pant et al. 1973; Salamonsen et al. 1973), was not found in the control animals in the present study.

Since oestradiol has been shown to induce oestrous behaviour in progesterone-primed ovariectomized ewes (Robinson 1959; Scaramuzzi et al. 1971), the reduction in the incidence of/

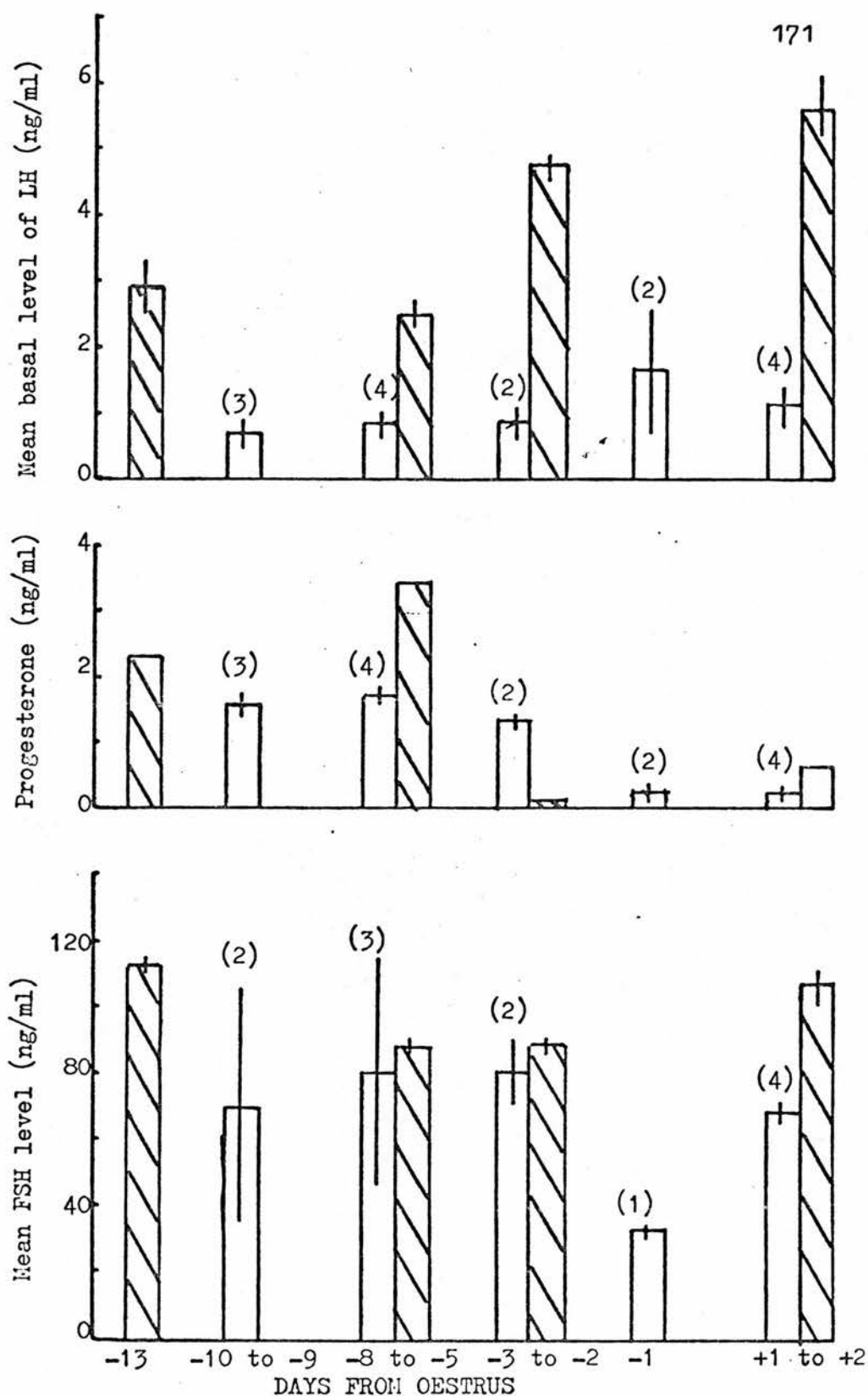


Figure 5.15 The concentration of progesterone and FSH and mean basal level of LH during the oestrous cycle in the control ewes and an oestradiol-immunized animal (9R-625).

The open bar represents the control value and the hatched bar the oestradiol-immunized value.

The vertical bar represents the range (mean  $\pm$  S.E.). The number in parentheses represents the number of control animals.

of oestrus in four oestradiol-immunized ewes following the primary immunization and the failure of these same animals to show oestrus after treatment with progesterone, indicated that the level of biologically active oestradiol had been reduced below the threshold required for the expression of oestrous behaviour. In contrast, the regular oestrous cycles shown by the fifth oestradiol-immunized ewe following the primary immunization and the occurrence of a normal oestrous cycle, albeit at a later time after progesterone treatment compared to the control animals, suggested the presence of a significant amount of biologically active oestradiol in this animal.

Passive immunization with antisera to oestradiol will also prevent the expression of oestrous behaviour in ovariectomized ewes in response to oestradiol (Scaramuzzi, 1975a) and in intact, cycling ewes (Fairclough et al. 1976 a,b). Similarly, active immunization against oestrogens will disrupt the oestrous cycle in the rat (Hillier et al. 1975a,b) and the menstrual cycle in the rhesus monkey (Cowchock et al. 1973; Sundaram et al. 1973).

The elevated level of LH in the oestradiol-immunized ewes indicated a reduction in the negative feedback control of LH secretion as discussed earlier. A similar elevation in the level of LH was found in anovulatory oestradiol-immunized rhesus monkeys (Ferin et al. 1974) and rats (Hillier et al. 1975a,b). A similar conclusion may be drawn for the elevated FSH levels in two oestradiol-immunized ewes.

Passive immunization studies with oestradiol antisera have demonstrated the involvement of oestradiol in the ovulatory/

ovulatory process in rats (Ferin et al. 1969a,b) and sheep (Fairclough et al. 1976a). Since active immunization against oestradiol will prevent the LH surge in ovariectomized ewes in response to oestradiol (Scaramuzzi et al. 1970a), it is likely that in the anoestrous oestradiol-immunized ewes, the presence of high titres of oestradiol antibodies had neutralized the peak of oestradiol secretion occurring some 20-30 hours before the onset of oestrus (Moore et al. 1969; Scaramuzzi et al. 1970b; Baird et al. 1976a) thereby preventing the pre-ovulatory surge of LH and subsequent ovulation. The absence of corpora lutea in the ovaries from the anoestrous oestradiol-immunized ewes (discussed elsewhere in this chapter) confirmed the anovulatory condition in these animals.

The concentration of progesterone during the oestrous cycle from the cycling oestradiol-immunized ewe followed a similar pattern to that found in the control animals. Since progesterone is secreted solely by the corpus luteum in the ewe (Short et al. 1963; Moore et al. 1969; Baird et al. 1975) and is directly proportional to the number of corpora lutea (Short, 1961; Thorburn et al. 1969), it is likely that this animal had ovulated. It is evident, therefore, that whilst a reduction in the level of biologically active oestradiol reduced the negative feedback control of tonic or basal LH secretion, the level of biologically active oestradiol secreted immediately before oestrus was sufficient to induce oestrous behaviour and the release of the preovulatory LH surge.

The present study served to verify the reduction in the negative/

negative feedback control of gonadotrophin secretion due to a reduction in the level of biologically active oestradiol seen during anoestrus. The fact that the levels of LH and FSH in the oestradiol-immunized ewes were higher during the breeding season than during anoestrus even though the antibody titres were lower during the breeding season, seemed to confirm the hypothesis of Karsch et al. (1976) that the hypothalamo-hypophyseal axis was more sensitive towards oestradiol during anoestrus.

#### 5.4. THE OVARIAN MORPHOLOGY AND STEROID CONCENTRATIONS IN EWES IMMUNIZED AGAINST E<sub>2</sub>-6-BSA.

##### 5.4.1. PROCEDURE.

The four oestradiol-immunized ewes which failed to show behavioural oestrus were laparotomized 17 days after the previous experiment (i.e. on day 10 of the cycle had they shown a normal 17-day cycle). The fifth ewe (9R-625) was laparotomized 7 days after the final sampling period in the previous experiment (i.e. day 9 of the oestrous cycle).

The ewes were laparotomized as described previously. The ovaries were exposed and the number and diameter of corpora lutea and large follicles ( $\geq 5$ mm diameter) were noted. In contrast to the method of collection of ovarian venous blood described earlier, 10-25ml of blood was drawn into a syringe directly from the largest vein draining each ovary. Follicular fluid was aspirated from the large follicles with a 1ml syringe. A sample of jugular venous blood (40-50ml) was/

was obtained by venipuncture at the same time as the ovarian blood collection. The blood samples were centrifuged and the plasma, together with the follicular fluids, stored frozen until assayed.

The ovaries and adrenal glands were removed after the collection of blood samples and follicular fluids. The corpora lutea were removed from the remainder of the ovary. The tissues were weighed and the ovarian tissues and adrenals fixed in Bouin's and Helly's fixatives respectively, as described earlier. The ovaries and uterus from one oestradiol-immunized ewe (OR-872) were removed together and fixed in formol-saline.

The animals were killed with an overdose of anaesthetic after the experiment.

#### 5.4.2. RESULTS.

The titre of steroid antibodies in jugular venous plasma from the oestradiol-immunized ewes are shown in Table 5.7. Since the laparotomies were performed 17 days after the previous experiment, the antibody titres were similar to those described earlier (Table 5.5).

The weight of ovarian tissue together with the number of large ovarian follicles in the oestradiol-immunized ewes are shown in Table 5.8. The weight of non-luteal ovarian tissue in the four anovulatory oestradiol-immunized ewes was significantly higher than the control value. The number of large follicles (5-11mm and >11mm diameter), was also increased in these animals. To illustrate the ovarian enlargement/



TABLE 5.7.  
THE TITRE<sup>a</sup> OF STEROID ANTIBODIES IN OESTRADIOL-INNMUNIZED EWES

<u>CONTROL</u>	<u><sup>3</sup>H-OESTRADIOL</u>	<u><sup>3</sup>H-OESTRONE</u>	<u><sup>3</sup>H-TESTOSTERONE</u>	<u><sup>3</sup>H-ANDROSTENEDIONE</u>
	N.S.	N.S.	1:2	N.S.
<hr/>				
<u>OESTRADIOL-INNMUNIZED</u>				
OR-535	1:5000	1:2200	1:10	1:3
9R-727	1:3800	1:2700	1:50	1:4
9R-625	1:950	1:30	1:7	1:1
OR-606	1:1400	1:70	1:13	1:1
OR-872	1:6200	1:2000	1:200	1:6

<sup>a</sup>Titre = The initial dilution of plasma which bound 50% of 15-20pg of tritiated steroid.

N.S. = <50% binding of the tritiated steroid at a 1:1 dilution.



TABLE 5.8.  
THE OVARY IN OESTRADIOL-IMMUNIZED EWES

			Day of the cycle	Wt.C.L. (mg)	Wt.NLT (mg)	No.of follicles Diameter(mm)	
						5 - 11	>11
<u>IMMUNIZED</u>							
OR-606	RO	NBO	-	5026*	1	3	
	LO			1264	0	0	
9R-727	RO	NBO	-	3110*	0	4	
	LO			1970*	0	1	
OR-535	RO	NBO	-	13694*	0	4	
	LO		-	11982*	3	2	
OR-872	RO	NBO	A	A	3	6	
	LO		A	A	1	5	
9R-625	RO	9	1,411(3C.L.)	781	5	0	
	LO		-	658	1	0	
CONTROL			497	826	1.0 $\pm$ 0.8	0	
(M $\pm$ SD)			$\pm$ 126	$\pm$ 229	(no.per animal)		
n.			6	8	4		

C.L. = corpora lutea. NLT = non-luteal tissue - ovary after the removal of C.L. NBO = no behavioural oestrus.

RO = right ovary. LO = left ovary. A = ovary and uterus fixed in situ.

\* Value lies outside control mean + 2 SD.

enlargement and multiple follicular development, the ovaries and uterus from an oestradiol-immunized ewe (OR-872) are shown in figure 5.16. Large follicles of up to 40mm diameter were found in this animal.

The fifth oestradiol-immunized ewe (9R-625) showed an ovulation rate of 3 as determined by the presence of 3 corpora lutea. Furthermore, the weight of non-luteal ovarian tissue in this animal was similar to the control value (Table 5.8).

The concentrations of oestradiol, oestrone, androstenedione and testosterone in peripheral and ovarian venous plasma from the oestradiol-immunized ewes are shown in Table 5.9. The measurement of oestradiol in the ovarian venous plasma from the oestradiol-immunized ewes was of doubtful accuracy due to the very low recovery of exogenous oestradiol, even after protein precipitation with hydrochloric acid (P.F.A. Van Look and I. Swanston, personal communication) and these values have been omitted from Table 5.9. Nevertheless, the concentration of oestradiol in peripheral plasma from four oestradiol-immunized ewes was significantly higher than the control value (Table 5.9). The peripheral plasma sample from the fifth oestradiol-immunized ewe (OR-872) was inadvertently discarded before the steroid levels could be measured.

The concentration of oestrone in peripheral and ovarian venous plasma (Table 5.9) was higher in the 3 oestradiol-immunized ewes with relatively high oestrone antibody titres (OR-872, OR-535, 9R-727; Table 5.7) reflecting the presence of high affinity oestrone antibodies.

The/

Figure 5.16.

The ovaries and uterus from an oestradiol-immunized  
ewe (OR-872).

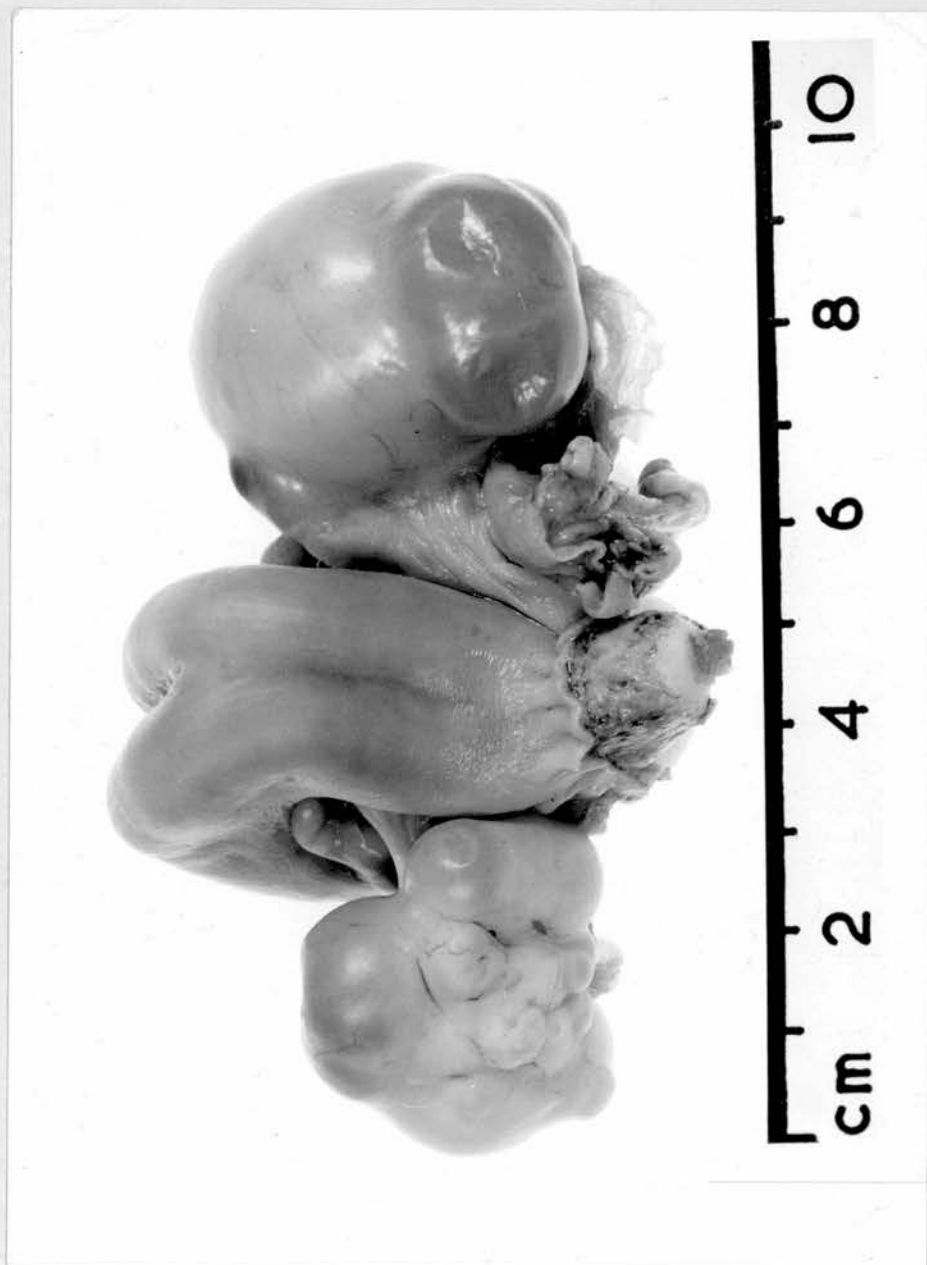


TABLE 5.9.

THE CONCENTRATION OF OESTRADIOL, OESTRONE, ANDROSTENEDIONE AND TESTOSTERONE  
IN PERIPHERAL AND OVARIAN VENOUS PLASMA FROM OESTRADIOL-IMMUNIZED EWES

	Oestradiol (ng/ml)			Oestrone (ng/ml)			Androstenedione (ng/ml)			Testosterone (ng/ml)		
	P	RO	LO	P	RO	LO	P	RO	LO	P	RO	LO
IMMUNIZED												
OR-606	0.310*	-	-	0.037*	0.073	0.093	0.135	0.893*	0.704*	0.121*	0.249	0.224
OR-872	N.S.	-	-	N.S.	0.784*	0.877*	N.S.	23.7*	29.0*	N.S.	0.384	0.412
9R-727	> 1.2*	-	-	N.S.	1.280*	N.S.	0.371	47.7*	N.S.	0.140*	0.957*	N.S.
OR-535	1.4*	-	-	0.040*	0.229*	0.205*	0.397	2.806*	3.063*	0.036	0.122	0.153
9R-625	0.092*	-	-	0.002	0.082	0.012	< 0.020	0.492	0.492	< 0.020	0.254	0.254
CONTROL	0.033	0.052		0.019	0.096		0.125	0.315		0.044	0.186	
(M±SD)	+0.008	+0.032		+0.005	+0.043		+0.143	+0.151		+0.030	+0.132	

P = peripheral. RO = right ovary. LO = left ovary. N.S. = no sample.

\*Value lies outside corresponding control mean  $\pm 2$  S.D.

The concentration of androstenedione in ovarian venous plasma from four oestradiol-immunized ewes was significantly higher than the corresponding control value (Table 5.9). However, the peripheral concentration of androstenedione in these animals lay within the control range (Table 5.9). The level of androstenedione in both peripheral and ovarian venous plasma from the fifth oestradiol-immunized ewe (9R-625) lay within the range of the corresponding control values (Table 5.9).

Although the concentration of testosterone in ovarian venous plasma from the oestradiol-immunized ewes was essentially within the range of the control values, the peripheral level of testosterone in two ewes (OR-606, 9R-727; Table 5.9) was about 3 times higher than the mean control level suggesting that in these animals a reduction in the clearance rate, presumably due to steroid-antibody binding, had occurred.

The concentration of progesterone in peripheral and ovarian venous plasma from the oestradiol-immunized ewes is shown in Table 5.10. High levels of progesterone were present in the ovarian venous plasma from the anovulatory oestradiol-immunized ewes suggesting that a number of luteinized follicles may have been present. The level of progesterone in ovarian venous plasma from the cycling oestradiol-immunized ewe (9R-625) was similar to that found in ovarian venous plasma from the control animals (Table 5.10).

The binding of 15-20pg of tritiated oestradiol by a 1/200 dilution of follicular fluid, determined by charcoal absorption, together with the percentage of oestradiol bound by/

TABLE 5.10.  
THE CONCENTRATION OF PROGESTERONE IN PERIPHERAL AND  
OVARIAN VENOUS PLASMA FROM OESTRADIOL-IMMUNIZED EWES.

	<u>Progesterone (ng/ml)</u>		
	P	RO	LO
<u>IMMUNIZED</u>			
OR-606	4.8*	551(0)	435(0)
OR-872	1.7*	417(0)	267(0)
9R-727	0.4*	266(0)	N.S.
OR-535	0.8*	56(0)	513(0)
9R-625	3.5	987(3)	9.2(0)
<u>CONTROL</u>			
P (M±SD)	3.1±0.5		
Ovary without C.L. (M±SD)		3.1±3.8	
n.		4	
Ovary with C.L. (M±SD)		582±263	
n.		4	

P = peripheral. RO = right ovary. LO = left ovary

C.L. = corpora lutea.

( ) = number of C.L.

n = number of observations.

\*Value lies outside corresponding control mean ± 2 S.D.

by a 1/200 dilution of peripheral plasma from four oestradiol-immunized ewes are shown in Table 5.11. The percentage of oestradiol bound by follicular fluid from the three anoestrous oestradiol-immunized ewes was similar to the corresponding values in peripheral plasma. Although appreciable oestradiol binding was present in the follicular fluid from the cycling oestradiol-immunized ewe (9R-625; Table 5.11), the value was beneath the steroid binding found in peripheral plasma.

#### 5.4.3. DISCUSSION.

The absence of corpora lutea in the ovaries from the four anoestrous oestradiol-immunized ewes indicated an anovulatory condition in these animals. Furthermore, the increased weight of ovarian tissue and the presence of large follicles of up to 40mm diameter reflected increased gonadotrophic stimulation due to the elevated levels of circulating gonadotrophins described previously. A similar anovulatory condition, accompanied by ovarian hypertrophy with multiple cystic follicles has been described in oestradiol-immunized rats (Hillier et al. 1975a,b) and rhesus monkeys (Ferin et al. 1974).

The absence of ovarian hypertrophy and multiple follicular development in the cycling oestradiol-immunized ewe reflected the lower gonadotrophin levels in this animal compared to the other oestradiol-immunized ewes. The ovulation rate in this animal was higher than the control value and may reflect, as suggested in the androstenedione-immunized ewes, a lower rate of/



TABLE 5.11

THE BINDING OF 15-20pg OF  $^3\text{H}$ -OESTRADIOL BY A 1/200

DILUTION OF FOLLICULAR FLUID AND PERIPHERAL

PLASMA FROM THE OESTRADIOL-IMMUNIZED EWES.

	Follicle diameter (mm)	% of $^3\text{H}$ -oestradiol bound
OR-606	P	86.0
	20	83.5
	18	85.8
	12	84.6
9R-727	P	85.0
	25	78.7
	15	73.6
	15	80.4
	15	74.2
	15	78.2
OR-535	P	86.0
	28	69.1
	27	68.8
	25	68.7
	5	81.5
9R-625	P	80.0
	10	37.9
	5	39.1

P = Peripheral plasma

of follicular atresia rather than a stimulation of follicular growth.

The elevated concentration of oestradiol in jugular venous plasma from the oestradiol-immunized ewes reflected the presence of high affinity antibodies, thereby reducing the clearance rate of this steroid. The magnitude of the elevation of the peripheral oestradiol concentration was correlated to the titre of oestradiol antibodies.

In contrast, the concentration of androstenedione in the peripheral plasma from the anovulatory oestradiol-immunized ewes was not significantly elevated even though the concentration of this steroid in ovarian venous plasma was very much higher than the corresponding control value. This finding suggests that although the ovarian secretion of androstenedione was stimulated, the absence of high affinity binding of this steroid, indicated by the low androstenedione antibody titres, failed to alter the metabolic clearance rate of androstenedione thereby resulting in relatively normal peripheral levels of this steroid. Since LH has been shown to stimulate the secretion of both oestradiol and androstenedione by the sheep ovary (Baird et al. 1968b, 1976b), it is likely that the elevated level of LH in the anovulatory oestradiol-immunized ewes had stimulated the secretion of androstenedione, and probably oestradiol although the ovarian venous concentration of the latter steroid was not determined.

In contrast, the concentrations of oestrone, androstenedione and testosterone in peripheral and ovarian venous plasma from the cycling oestradiol-immunized ewe were within/

within the control range. It may be concluded that in this animal, ovarian steroid secretion was normal and the marginal elevation in the concentration of oestradiol in peripheral plasma may be attributed to the presence of a low titre of high affinity antibodies.

The concentration of testosterone in ovarian venous plasma from the anovulatory oestradiol-immunized ewes was essentially unaltered, indicating that the ovarian secretion of this steroid was not stimulated. It is interesting to note that the peripheral concentration of testosterone was elevated in two oestradiol-immunized ewes. If this elevation was due to a decreased clearance rate brought about by steroid-antibody binding, then it is likely that the level of biologically active androgen was reduced. In these same animals, the concentration of FSH was not elevated compared to the control value. It is possible, therefore, that, as suggested earlier, the absence of androgen may have increased the negative feedback action of oestradiol on FSH secretion.

#### 5.5. CHAPTER SUMMARY.

1. All five ewes responded to active immunization with  $E_2$ -6-BSA by the production of significant titres of oestradiol antibodies. Elevated binding of oestrone and testosterone, and, to a lesser extent, androstenedione, was also present.
2. Four of the oestradiol-immunized ewes showed a reduction in the incidence of oestrus following the primary immunization and failed to show oestrus after treatment with progesterone. The fifth animal continued to show regular oestrous cycles following/

following the primary immunization and after progesterone treatment. These findings suggest that the level of biologically active oestradiol was reduced in the anoestrous ewes to below the threshold required for the expression of oestrous behaviour.

3. The frequency of spontaneous LH peaks and the basal level of LH during anoestrus was increased in the oestradiol-immunized ewes compared to the control animals and approached those values seen in ovariectomized-hysterectomized ewes. The present results confirm the hypothesis that oestradiol is the major ovarian steroid involved in the negative feedback control of LH secretion in the ewe during anoestrus.

The significant elevation in the concentration of FSH during anoestrus in two oestradiol-immunized ewes suggested that oestradiol exerts a negative feedback action on FSH secretion in the ewe. A similar elevation in the level of FSH occurred in the absence of all ovarian steroids (i.e. in ovariectomized ewes).

The finding that normal FSH levels were present in three oestradiol-immunized ewes with relatively low oestradiol antibody titres suggested that the neutralization of oestradiol was incomplete and a sufficient amount of biologically active oestradiol was available to exert a negative feedback action on the release of FSH. An alternative hypothesis was that the level of biologically active androgen was reduced in these animals thereby enhancing the negative feedback action of oestradiol.

4. During the breeding season, the concentration of LH was elevated in the four anoestrous oestradiol-immunized ewes.

This/

This finding suggested that oestradiol plays an important role in the negative feedback control of LH during the oestrous cycle. The absence of corpora lutea confirmed the anovulatory condition of these animals. It is likely that the presence of oestradiol antibodies had neutralized the peak of oestradiol occurring 20-30 hours before the onset of oestrus in the intact ewe, thereby preventing oestrous behaviour and the preovulatory surge of LH. The presence of enlarged follicles in the ovaries from the anovulatory ewes reflected the elevated levels of circulating gonadotrophins.

A normal oestrous cycle occurred in the fifth oestradiol-immunized ewe. Since the ovarian weight in this animal was within the control range and the ovulation rate was higher than the control value, it was suggested that a reduction in the rate of follicular atresia rather than follicular stimulation had occurred.

5. The presence of elevated concentrations of oestradiol in peripheral plasma from the oestradiol-immunized ewes indicated the presence of high affinity antibodies.

The presence of elevated concentrations of androstenedione in ovarian venous plasma accompanied by normal levels in peripheral plasma from the four anovulatory ewes suggested that although the elevated LH levels had stimulated ovarian steroid secretion, the clearance rate of androstenedione was unaltered due to the absence of androstenedione antibodies.

The presence of elevated levels of testosterone in two oestradiol-immunized ewes suggested the presence of high affinity testosterone antibodies. If androgens enhance the release of FSH/

FSH, then the lower level of biologically active testosterone may have resulted in a reduction in the release of FSH in these animals.

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CHAPTER 6

THE RELEASE OF LH AND FSH AND THE CONCENTRATION OF  
OVARIAN STEROIDS IN EWES ACTIVELY IMMUNIZED AGAINST  
TESTOSTERONE-3-(O-CARBOXYMETHYL)OXIME-BOVINE SERUM ALBUMIN

## 6.1. INTRODUCTION

Although testosterone is secreted by the ovary in sheep (Baird et al. 1968b; 1973), its physiological role, if any, has not been described.

High doses of testosterone propionate (1mg or greater) have been shown to induce oestrous behaviour in ovariectomized ewes (Lindsay & Robinson, 1961, 1964) and may cause ovulation in lactating (Restall, Radford & Wallace, 1972) and anoestrous (Radford & Wallace, 1971) ewes. It is likely that these doses of testosterone will produce supra-physiological levels and the effects described above may well have been due to extraovarian aromatization of testosterone to oestradiol rather than a direct action of testosterone itself.

The present study attempted to neutralize the circulating level of biologically active testosterone by active immunization against testosterone-3-(o-carboxymethyl) oxime-bovine serum albumin (T-3-BSA).

Five Welsh mountain ewes were actively immunized with 2.0mg of T-3-BSA during November, 1974. Booster immunizations with 2.0mg and 0.6mg of the conjugate were given after 101 and 266 days respectively.

Following the primary immunization, the incidence of oestrus decreased in four of the testosterone-immunized ewes (fig. 6.1). The fifth animal (9R-443), however, continued to show regular oestrous cycles (fig. 6.1).

## 6.2./



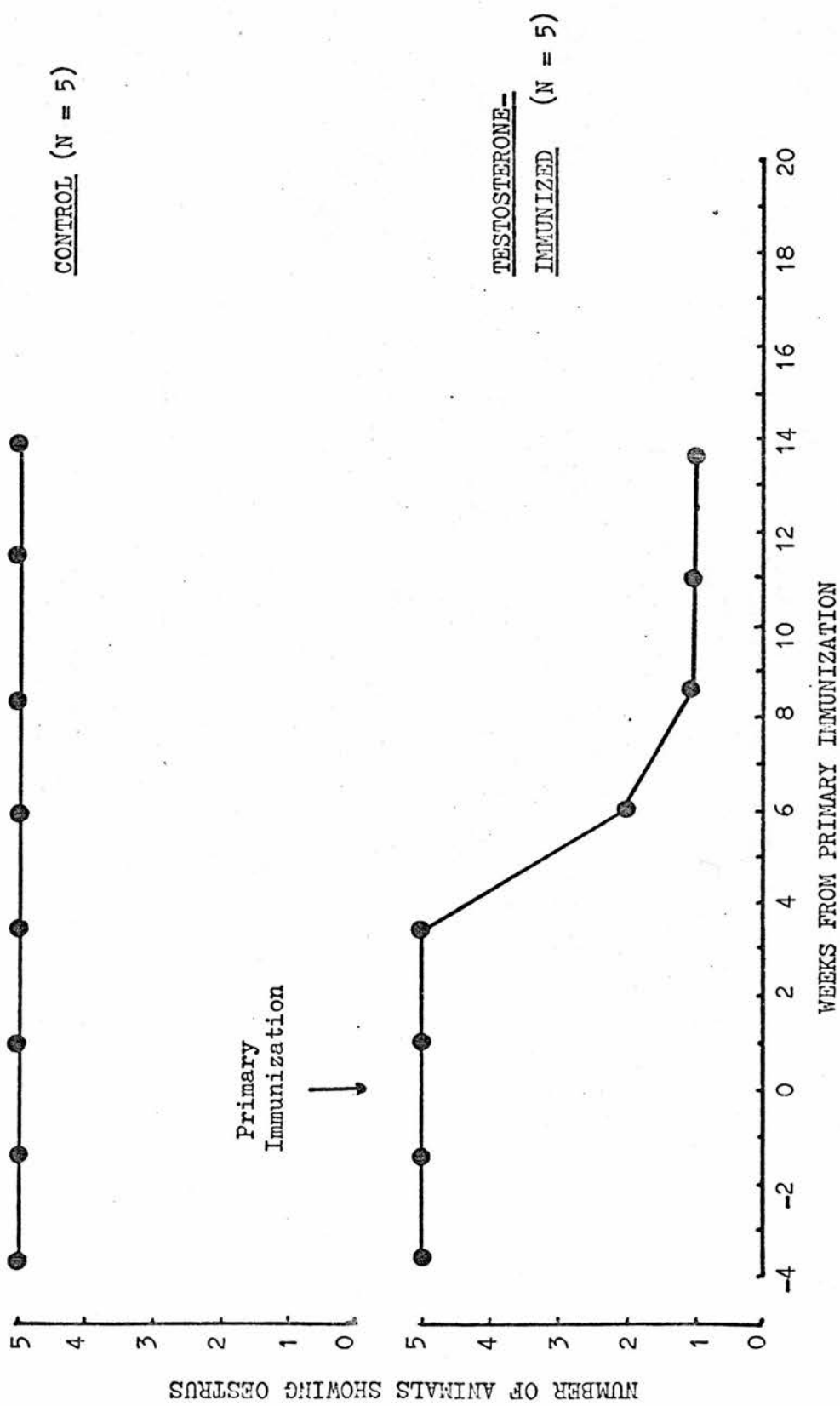


Figure 6.1. The incidence of oestrus in control and testosterone-immunized ewes following the primary immunization.

6.2. THE EFFECTS OF IMMUNIZATION AGAINST T-3-BSA ON THE  
RELEASE OF LH AND FSH DURING ANOESTRUS IN THE EWE.

6.2.1. PROCEDURE.

An investigation into the effects of immunization against T-3-BSA on the plasma concentrations of LH and FSH in the ewe during anoestrus was performed in conjunction with the study of gonadotrophin levels during anoestrus in oestradiol-immunized ewes described in Chapter 5 (section 5.2).

The present experiment was performed in August 1975, 14 days after the testosterone-immunized ewes had received their second booster immunization.

Blood samples (5ml) were obtained by indwelling jugular vein cannulae at 15 minute intervals for 12 hours (1300-0100h). The testosterone-immunized ewes then received an intramuscular injection of either 25 $\mu$ g or 50 $\mu$ g of oestradiol benzoate (OB) (in 1ml of arachis oil) and sampling continued at 2-hourly intervals for 48 hours.

One week later, serial blood samples were again taken from the testosterone-immunized ewes at hourly intervals for 6 hours (2000-0100h). Each animal then received an intramuscular injection of either 25 $\mu$ g or 50 $\mu$ g of OB (the opposite to the previous dose of OB) at 0100h and sampling proceeded at 2-hourly intervals for 48 hours.

The concentration of LH was determined in all samples taken from each animal throughout the experiment. The level of FSH was determined in alternate samples from each of/

of 3 testosterone-immunized ewes.

Plasma taken from each animal was pooled and the steroid antibody titres, together with the percentage of steroids bound after equilibrium dialysis, measured in each pooled sample.

#### 6.2.2. RESULTS.

The steroid antibody titres, determined by charcoal absorption, in the control and testosterone-immunized ewes are shown in Table 6.1. High titres of testosterone antibodies, ranging from 1:21000 to 1:49000, were present in plasma from the testosterone-immunized ewes. Significant titres of androstenedione (range: 1:390 - 1:580) and oestradiol (range: 1:390 - 1:630) antibodies were also encountered in the testosterone-immunized animals. The titre of oestrone antibodies (range: 1:9 - 1:27), although higher than the binding of oestrone by plasma from the control ewes, was lower than the titre of other steroid antibodies in the testosterone-immunized animals.

The mean percentages of steroids bound by plasma from the control and testosterone-immunized ewes, determined by equilibrium dialysis, are shown in Table 6.2. The percentage of all four steroids bound by plasma from the testosterone-immunized animals was significantly higher than the corresponding control value (Table 6.2).

The patterns of LH and FSH release during the 12-hour period in the control ewes has been described elsewhere (Chapter 5, section 5.2). In brief, the level of LH was low, with/

TABLE 6.1.  
THE TITRE<sup>a</sup> OF STEROID ANTIBODIES IN TESTOSTERONE-IMMUNIZED EWES

CONTROL	<sup>3</sup> H-TESTOSTERONE	<sup>3</sup> H-ANDROSTENEDIONE	<sup>3</sup> H-OESTRADIOL	<sup>3</sup> H-OESTRONE
	1:1	N.S.	N.S.	N.S.
<hr/>				
TESTOSTERONE- IMMUNIZED				
9R-443	1:21000	1:440	1:395	1:15
OR-550	1:35000	1:390	1:630	1:27
9R-451	1:42000	1:690	1:390	1:10
9R-397	1:37000	1:580	1:470	1:9
9R-589	1:49000	1:580	1:490	1:14

<sup>a</sup>Titre = The initial dilution of plasma which bound 50% of 15-20pg of tritiated steroid.

N.S. = <50% binding of the tritiated steroid at a dilution of 1:1.

TABLE 6.2.  
THE PERCENTAGE (N $\pm$ S.E.) OF 15-25pg OF TRITIATED STEROID BOUND BY A 1/5 DILUTION OF PLASMA  
FROM THE CONTROL AND TESTOSTERONE-IMMUNIZED EWES DETERMINED BY EQUILIBRIUM DIALYSIS.

	$^3\text{H}$ -TESTOSTERONE	$^3\text{H}$ -ANDROSTENEDIONE	$^3\text{H}$ -OESTRADIOL	$^3\text{H}$ -OESTRONE
CONTROL	81.7 $\pm$ 1.1 (n=4)	57.2 $\pm$ 2.2 (n=4)	88.4 $\pm$ 1.2 (n=4)	80.1 $\pm$ 3.1 (n=4)
TESTOSTERONE- IMMUNIZED	99.7 $\pm$ 0.1* (n=5)	99.5 $\pm$ 0.1* (n=5)	99.2 $\pm$ 0.3* (n=5)	98.6 $\pm$ 0.4* (n=5)

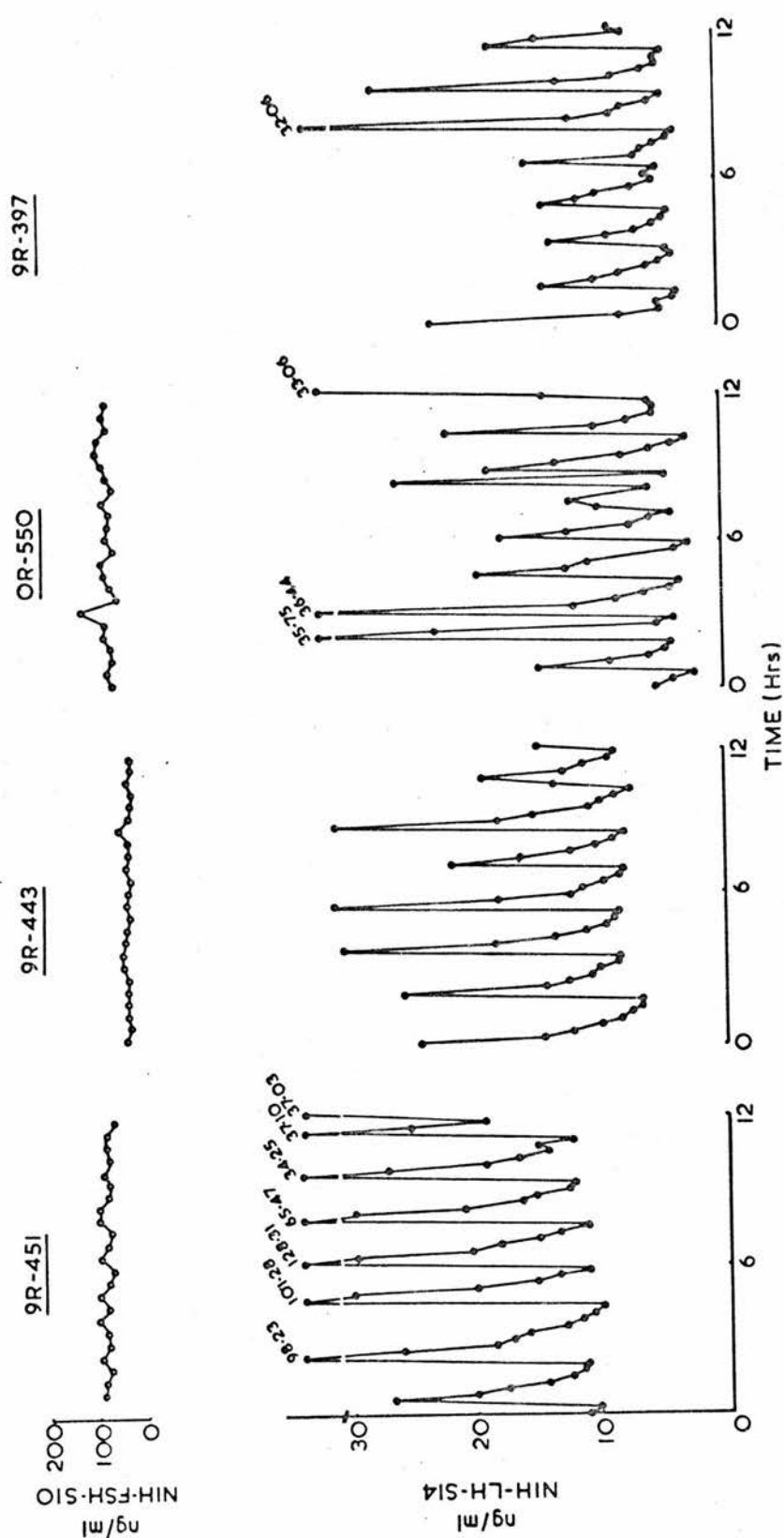
\* p<0.001. Immunized vs. Control (Student's t-test).

with the basal level ranging from 0.23 to 0.64ng/ml and one spontaneous discharge of LH (mean peak height ( $\pm$  S.E.):  $3.59 \pm 0.57$  ng/ml;  $n=4$ ) present in each animal. The concentration of FSH measured in 3 control ewes varied considerably (range, mean FSH level: 27.5 - 60.0 ng/ml) although little variation was apparent during the 12-hour period within each animal.

The concentrations of LH in plasma samples taken at 15 minute intervals for 12 hours from the five testosterone-immunized ewes are shown in figures 6.2 and 6.3. A pulsatile pattern of LH release was apparent in all ewes with 7 to 9 peaks present in each animal during the 12 hour period. Both the mean peak height and the basal levels of LH in the testosterone-immunized ewes were significantly higher than the corresponding control values (fig. 6.4). The elevation in the concentration of LH could not be directly related to the titre of testosterone antibodies since the ewe with the highest antibody titre (9R-589, Table 6.1) showed the lowest level of LH (fig. 6.3). Similarly, no correlation between the titres of androstenedione or oestradiol antibodies and the level of LH was apparent.

In contrast to the elevated levels of LH found in the testosterone-immunized ewes, the concentration of FSH in three of these animals (fig. 6.2) was similar to the levels of FSH found in the control animals (see fig. 5.2). The mean concentrations of FSH in the testosterone-immunized ewes, ranging from 42.8 to 87.6 ng/ml, were within the control range (mean  $\pm$  2S.D.) (fig. 6.5).

The basal levels of LH, determined by the mean of the five/



**Figure 6.2.** The plasma concentrations of LH and FSH during anoestrus in testosterone-immunized ewes. Samples were taken every 15 minutes for 12 hours. LH was assayed in every sample. FSH was measured in alternate samples from 3 animals.

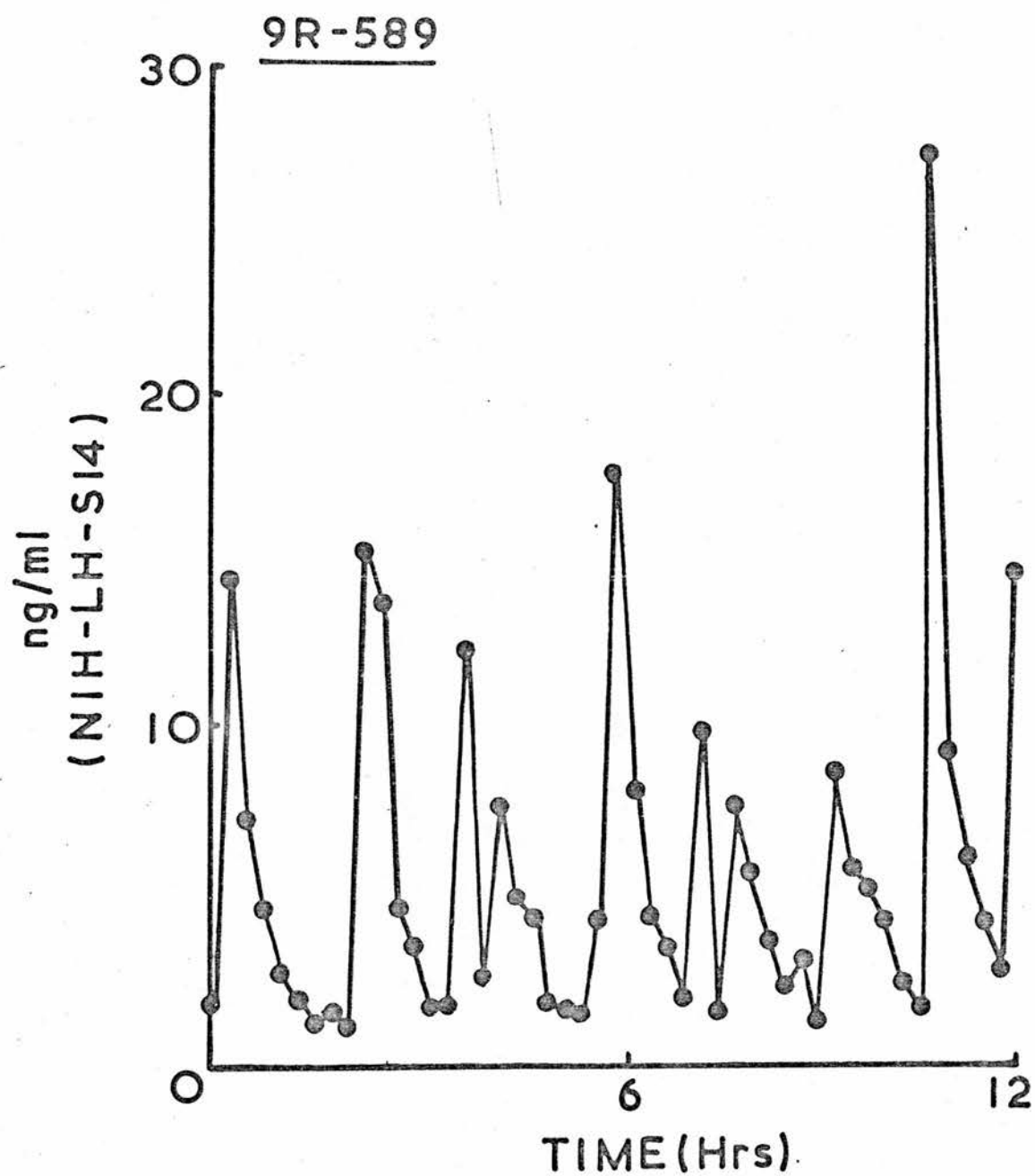


Figure 6.3. The plasma concentration of LH during anoestrus in a testosterone-immunized ewe.

Samples were taken at 15-minute intervals for 12 hours.  
LH was assayed in every sample.



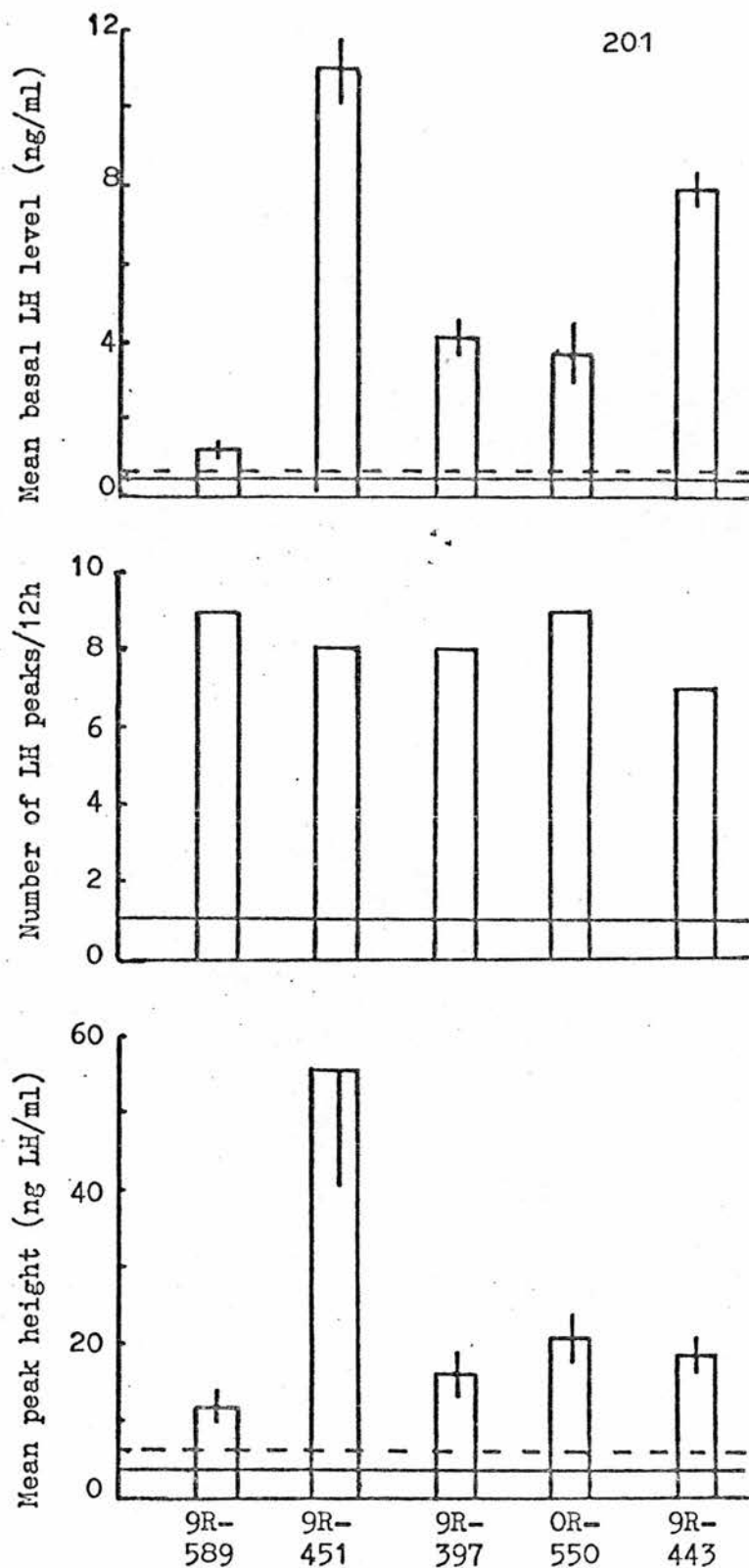


Figure 6.4. The pattern of LH release during anoestrus in control and testosterone-immunized ewes.

The values are arranged in order of decreasing testosterone antibody titres.

The vertical line represents the range (mean  $\pm$  S.D.) of individual animals. The horizontal line represents the mean and the broken line the range (mean + 2 S.D.) of the control group.

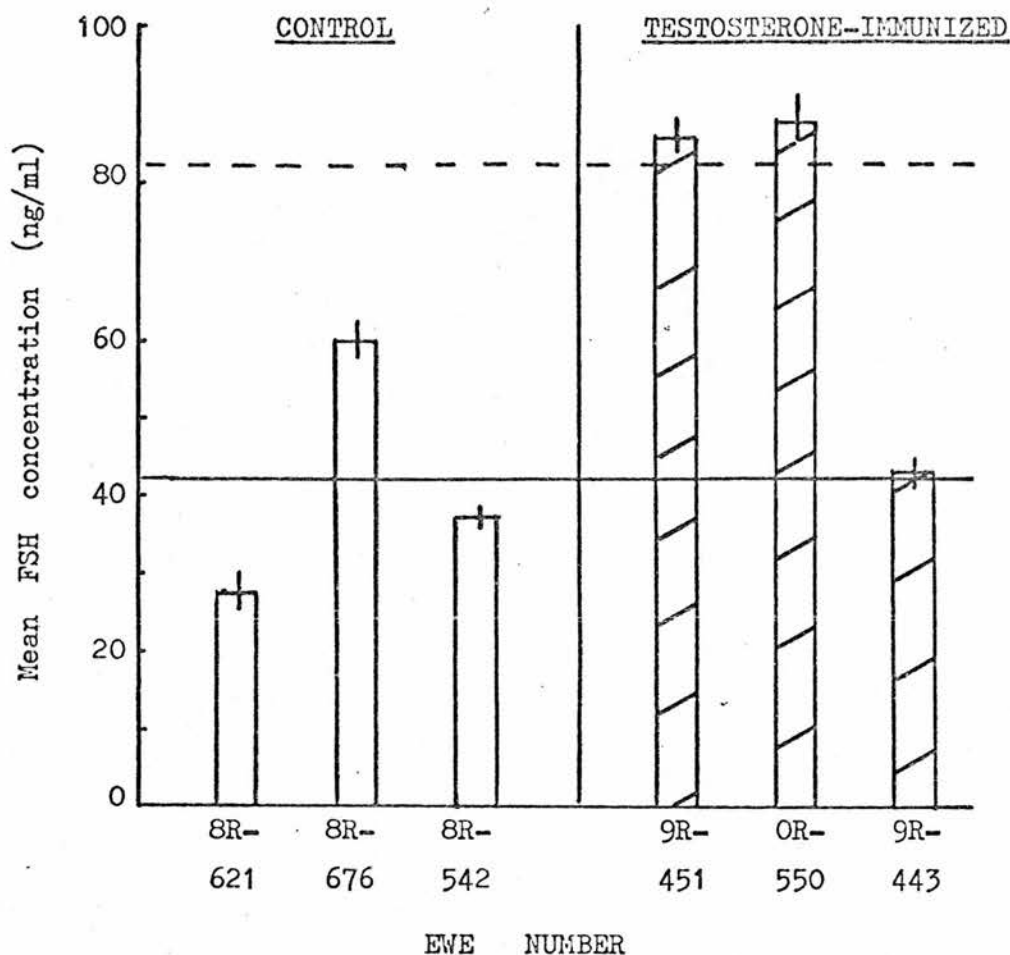


Figure 6.5. The mean concentration of FSH during anoestrus in control and testosterone-immunized ewes.

The testosterone-immunized animals are arranged in order of decreasing testosterone antibody titre.

The vertical line represents the range (mean  $\pm$  S.E.) in individual animals.

The horizontal line represents the mean, and the broken line the range (mean + 2 S.D.) of the control group.

five lowest concentrations of LH measured during the 48 hours following OB (25 $\mu$ g and 50 $\mu$ g) administration in the five testosterone-immunized ewes are shown in Table 6.3. The basal level of LH was not altered after OB administration. It was not possible to ascertain the number of LH peaks present during this period since the 2-hourly sampling frequency was greater than the duration of the spontaneous discharges of LH (1-2 hours) found during the 12-hour intensive sampling period. The positive feedback action of oestradiol on LH secretion was absent since at no time after OB administration in any of the testosterone-immunized ewes was the concentration of LH greater than the basal level ( $\pm$  2S.D.) for 5 consecutive samples (i.e. 10 hours).

Similarly the mean concentration of FSH following OB administration was not significantly different from the pre-injection value (Table 6.3). A positive feedback discharge of FSH was also absent since the level of FSH was not higher than the mean level ( $\pm$  2S.D.) for 2 or more consecutive measurements.

### 6.3. THE PLASMA CONCENTRATIONS OF LH, FSH AND PROGESTERONE DURING THE BREEDING SEASON IN TESTOSTERONE-IMMUNIZED EWES.

#### 6.3.1. PROCEDURE.

The present experiment was performed simultaneously with the investigation into the effects of active immunization against oestradiol on gonadotrophin levels during the breeding season described in Chapter 5 (section 5.3).

In/

TABLE 6.3.

THE MEAN CONCENTRATION OF FSH AND THE BASAL LEVEL  
OF LH IN TESTOSTERONE-IMMUNIZED EWES TREATED  
WITH OESTRADIOL BENZOATE (OB).

	$\mu\text{gOB}$	LH (ng/ml; M $\pm$ S.D.)		FSH (ng/ml; M $\pm$ S.D.)	
		Pre-OB	Post-OB	Pre-OB	Post-OB
OR-589	25 <sup>b</sup>	1.24 $\pm$ 0.18	1.28 $\pm$ 0.18	-	-
	50 <sup>a</sup>	-	2.02 $\pm$ 0.81		
9R-451	25 <sup>b</sup>	10.96 $\pm$ 0.78	10.70 $\pm$ 0.85	86.0 $\pm$ 9.6	86.8 $\pm$ 7.1
	50 <sup>a</sup>		9.64 $\pm$ 0.27		83.8 $\pm$ 7.9
9R-397	25 <sup>a</sup>	4.02 $\pm$ 0.50	3.60 $\pm$ 0.21	-	-
	50 <sup>b</sup>		3.35 $\pm$ 0.32		
OR-550	25 <sup>a</sup>	3.46 $\pm$ 0.72	3.72 $\pm$ 0.28	87.6 $\pm$ 15.3	92.9 $\pm$ 12.8
	50 <sup>b</sup>		4.11 $\pm$ 0.54		93.9 $\pm$ 14.5
9R-443	25 <sup>b</sup>	7.97 $\pm$ 0.39	8.25 $\pm$ 0.19	42.8 $\pm$ 5.2	44.8 $\pm$ 5.2
	50 <sup>a</sup>		7.29 $\pm$ 0.57		40.9 $\pm$ 3.7

Pre-OB - The pre-injection basal level of LH was determined by the mean of the 5 lowest concentrations of LH measured in samples taken at 15-minute intervals for 12 hours.

- The pre-injection level of FSH was determined by the mean concentration of FSH levels measured in alternate samples.

Post-OB - The post-injection basal LH level was determined by the mean of the 5 lowest concentrations of LH measured in samples taken at 2-hourly intervals for 48 hours following steroid administration. The post-injection level of FSH was determined by the mean concentration of FSH measured in alternate samples.

<sup>a</sup> The steroid was administered immediately following the 12-hour sampling period.

<sup>b</sup> The steroid was administered one week after the previous experiment.

In brief, the experiment was performed during September-October, 1975, 91 days after the testosterone-immunized ewes had received their second booster immunization.

Progesterone sponges were inserted into the vaginae of the nine control and testosterone-immunized ewes and remained in situ for 12 days. All the control ewes came into heat 3 to 4 days after the removal of the sponges and subsequently showed regular oestrous cycles of 15-17 days duration. None of the testosterone-immunized ewes were marked by the ram and remained in a state of anoestrus throughout the experimental period (i.e. within 29 days after the termination of progesterone treatment).

Serial samples (5ml) of jugular venous blood were obtained at 15-minute intervals for 6 hours from the four control ewes on each of 4 days during the oestrous cycle (see Table 5.4).

The five testosterone-immunized ewes were bled at 15-minute intervals for 6 hours on day "10" of the cycle, had they shown regular oestrous cycles in synchrony with the control animals.

The concentration of LH was measured in all samples, and the level of FSH determined in alternate samples taken from each of the testosterone-immunized ewes. The concentration of progesterone was determined in the first sample taken from each animal.

The steroid antibody titres were measured after pooling the samples taken from each animal.

### 6.3.2. RESULTS.

The steroid antibody titres in control and testosterone-immunized ewes are shown in Table 6.4. Since the present experiment was performed 91 days after a booster immunization compared to a 14-day interval in the anoestrus experiment, the antibody titres were lower than those described previously. Nevertheless, high titres of testosterone antibodies were present (range: 1:15000 - 1:44000). Significant titres of androstenedione and oestradiol antibodies, ranging from 1:44 to 1:700 and 1:16 to 1:320 respectively were also present in plasma from the testosterone-immunized ewes. Negligible binding of oestrone, however, was found (Table 6.4).

The concentrations of LH during the oestrous cycle in the control ewes have been discussed elsewhere (Chapter 5, section 5.3). The basal level of LH was low during the mid- and late-luteal stages of the cycle with higher concentrations of LH occurring in two animals sampled during the follicular phase. The concentration of progesterone in the control ewes followed a cyclic pattern with maximal levels during the luteal phase and a marked reduction during the follicular phase. Considerable variation in the concentration of FSH occurred between control ewes. A tendency for a lower level of FSH during the follicular phase of the cycle was observed in two animals.

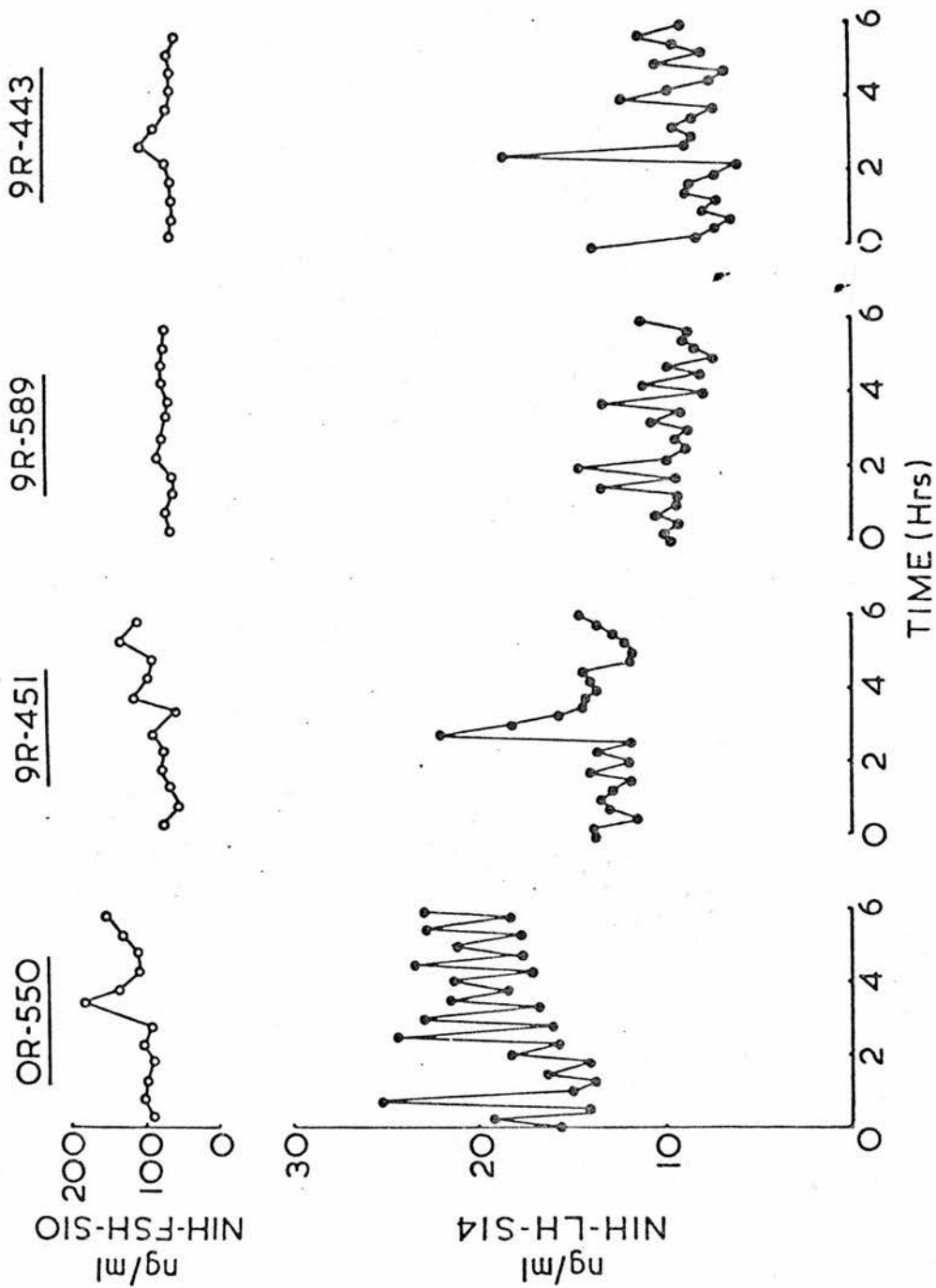
The plasma concentrations of LH and FSH during the breeding season in the five testosterone-immunized ewes are shown in figures 6.6 and 6.7. A marked elevation in the level/

TABLE 6.4.  
THE TITRE<sup>a</sup> OF STEROID ANTIBODIES IN TESTOSTERONE-IMMUNIZED EWES

CONTROL	<sup>3</sup> H-TESTOSTERONE	<sup>3</sup> H-ANDROSTENEDIONE	<sup>3</sup> H-OESTRADIOL	<sup>3</sup> H-OESTRONE
	1:2	N.S.	N.S.	N.S.
<hr/>				
TESTOSTERONE- IMMUNIZED				
9R-443	1:15000	1:44	1:47	N.S.
OR-550	1:23000	1:128	1:320	1:8
9R-451	1:20000	1:700	1:16	1:2
9R-397	1:21000	1:104	1:150	N.S.
9R-589	1:44000	1:500	1:150	1:6

<sup>a</sup>Titre = The initial dilution of plasma which bound 50% of 15-20pg of tritiated steroid.

N.S. = <50% binding of the tritiated steroid at a dilution of 1:1.



**Figure 6.6.** The plasma concentrations of LH and FSH during the breeding season in four testosterone-immunized ewes.

Samples were taken at 15-minute intervals for 6 hours. LH was assayed in every sample. FSH was measured in alternate samples.



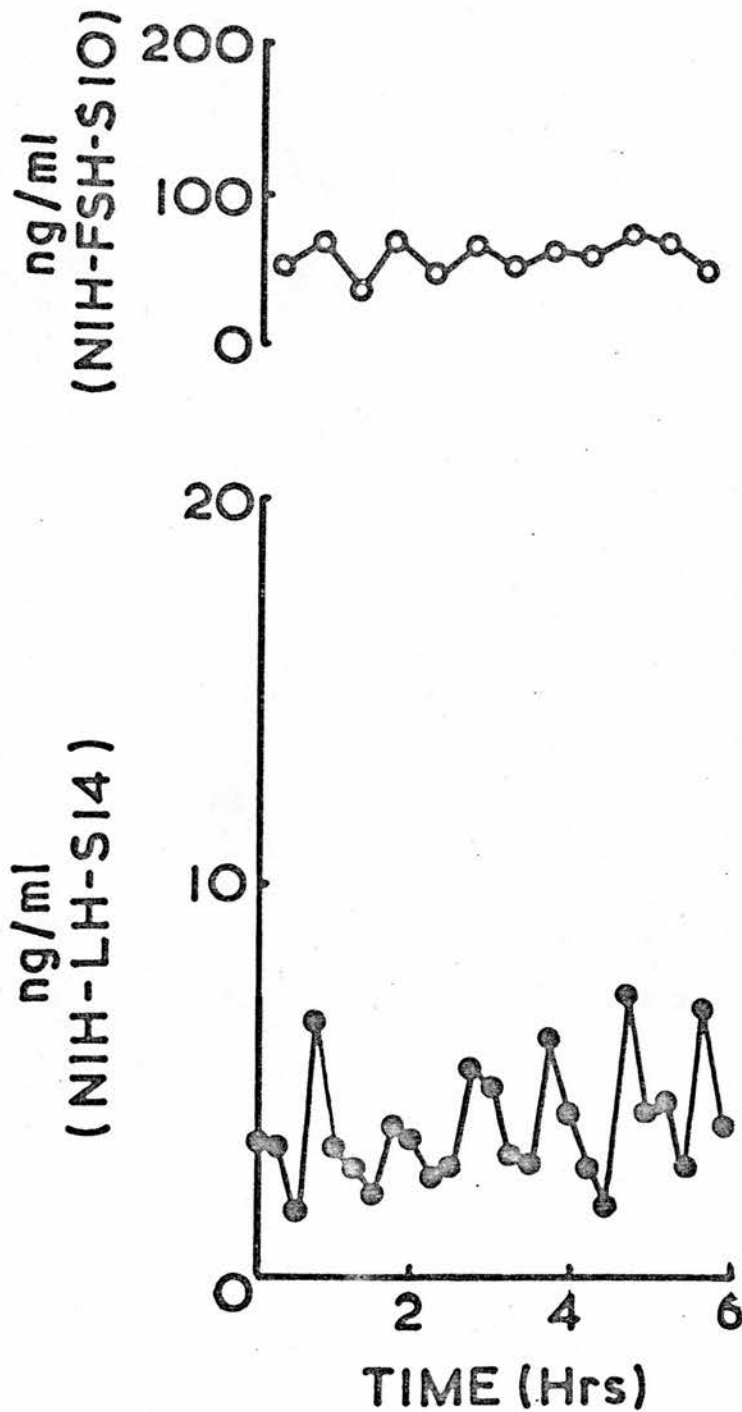
9R-397

Figure 6.7. The plasma concentrations of LH and FSH during the breeding season in a testosterone-immunized ewe.

Samples were taken at 15-minute intervals for 6 hours. LH was assayed in every sample. FSH was measured in alternate samples.

level of LH was found in 4 out of 5 testosterone-immunized ewes (fig. 6.6). The level of LH in the fifth ewe (fig. 6.7), however, was within the range of values found during the follicular phase of the cycle in control animals (fig. 6.8). In contrast, the level of FSH in the testosterone-immunized ewes lay within the range of values found in control animals during the oestrous cycle (fig. 6.9).

The concentration of progesterone in peripheral plasma from the testosterone-immunized ewes is shown in Table 6.5. The level of progesterone was low ( $< 1$  ng/ml) in four of the ewes. In the fifth ewe (9R-451), a high level of progesterone (7.4 ng/ml) was found.

#### 6.4. THE OVARIAN MORPHOLOGY AND STEROID CONCENTRATIONS IN THE TESTOSTERONE-IMMUNIZED EWES.

##### 6.4.1. PROCEDURE.

The testosterone-immunized ewes were subjected to two laparotomies. The five ewes were first laparotomized 15-16 days after the previous experiment. One testosterone-immunized ewe (9R-443) was marked by the ram on the day before the first laparotomy. The remaining four ewes had not shown oestrus since the termination of progesterone treatment, 105-106 days earlier.

Four testosterone-immunized ewes (one animal, 9R-443 had died from post-operative complications after the first laparotomy) received a booster immunization of 1.0mg of T-3-BSA 7 days after the first laparotomy. These animals were/

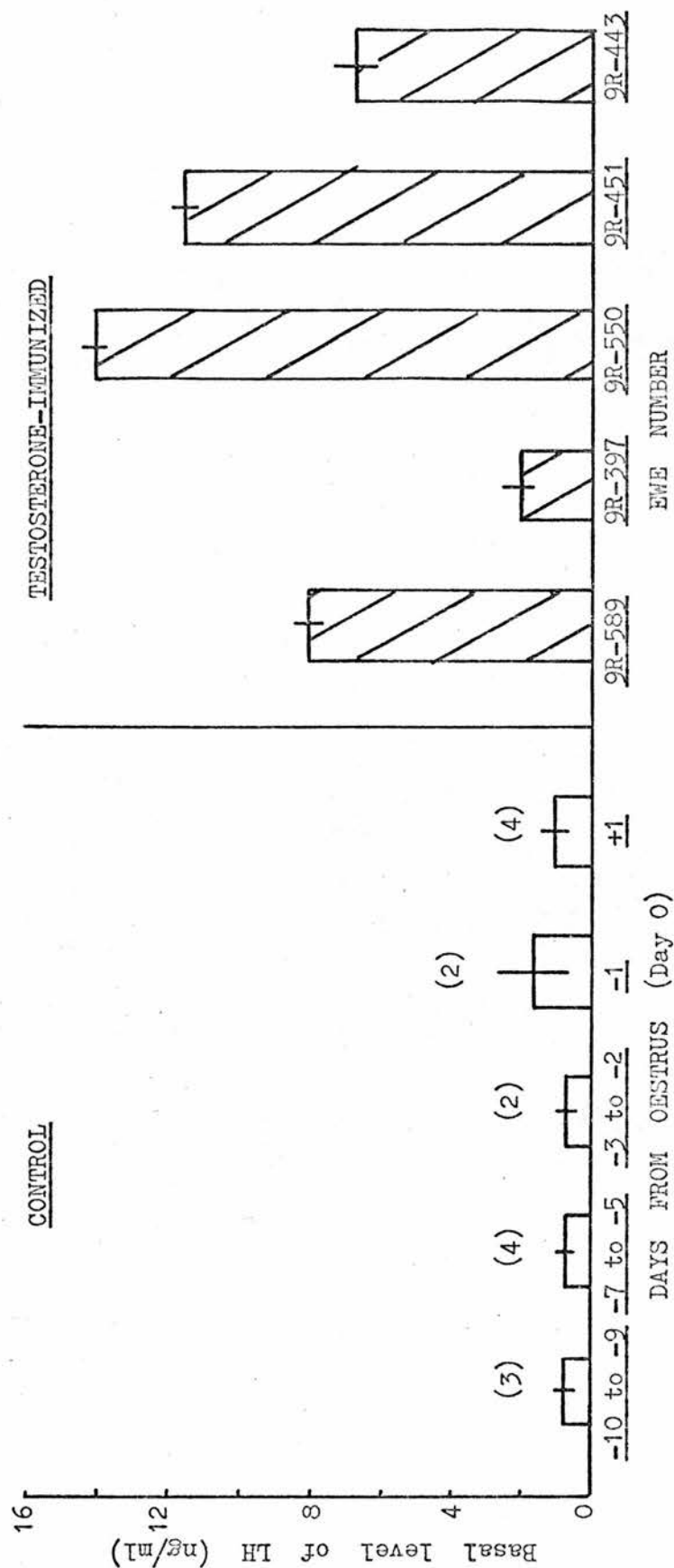


Figure 6.8. The basal level of LH during the breeding season in control and testosterone-immunized ewes.

The testosterone-immunized ewes are arranged in order of decreasing testosterone antibody titre. The vertical line represents the range (mean  $\pm$  S.D.). The numbers in parentheses represent the number of control animals.

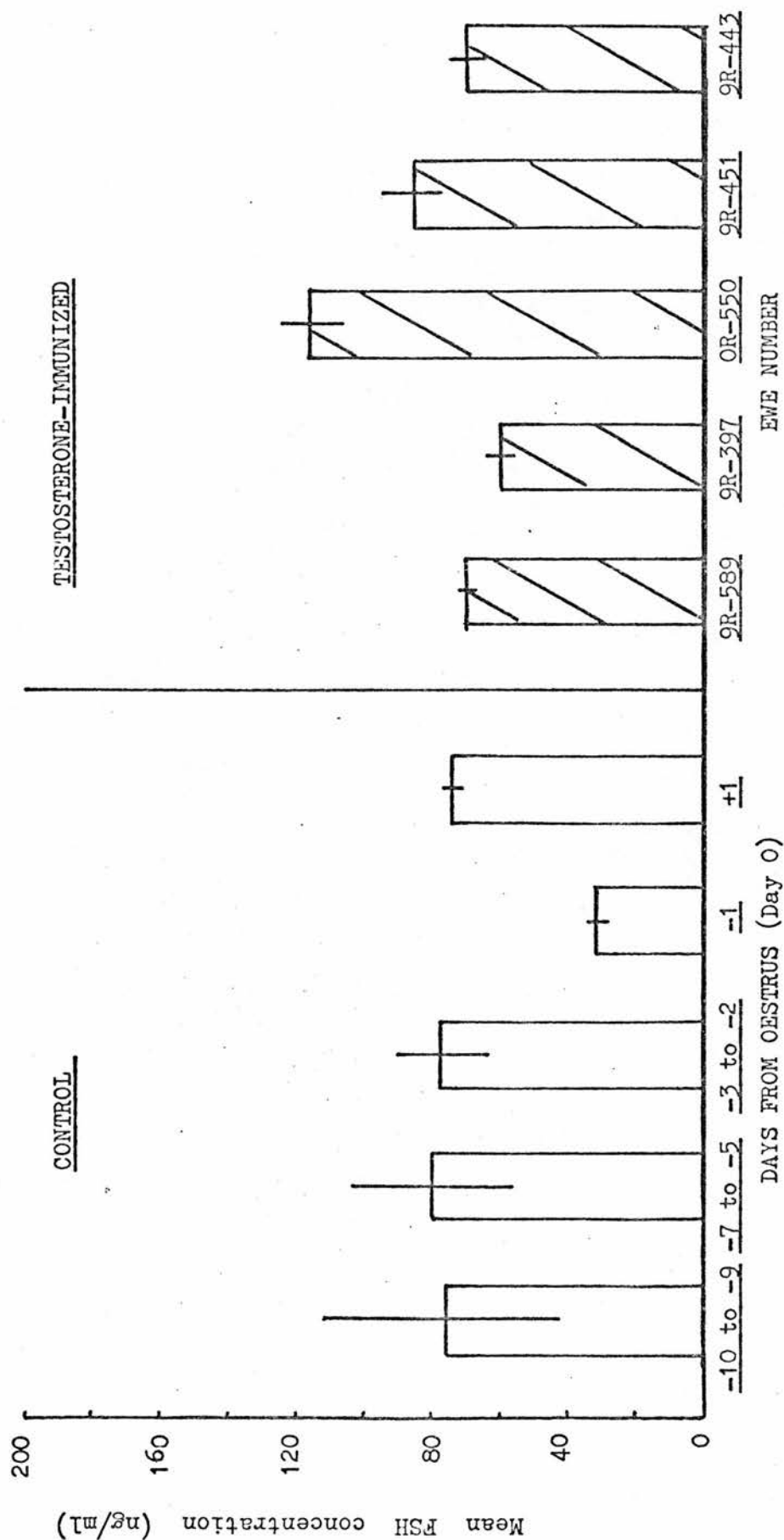


Figure 6.9. The mean concentration of FSH during the breeding season in control and testosterone-immunized ewes.

The vertical lines represent the range (mean  $\pm$  S.E.). The number in parentheses represents the number of control animals.

TABLE 6.5.

THE CONCENTRATION OF PROGESTERONE DURING THE BREEDING  
SEASON IN TESTOSTERONE-IMMUNIZED EWES.

					<u>Progesterone (ng/ml)</u>
9R-589	..	..	..	..	0.7
OR-550	..	..	..	..	0.7
9R-397	..	..	..	..	0.2
9R-451	..	..	..	..	7.4
9R-443	..	..	..	..	1.0

---

The concentration of progesterone was determined in the first sample taken from each ewe on the expected day "10" of the cycle had they shown oestrus in synchrony with the control animals.

were again laparotomized 6 or 26 days later.

The laparotomy procedure followed the method described previously (Chapter 5, section 5.4). A sample of ovarian venous blood was obtained from the largest vein draining each ovary. The number and diameter of corpora lutea and follicles ( $\geq 5$ mm diameter) were noted and follicular fluid was aspirated from the large follicles. A sample of jugular venous blood was obtained by venipuncture at the same time as the ovarian collections.

At the completion of the first laparotomy, the wound was closed and sutured and the animals allowed to recover. The ovaries were removed at the completion of the second laparotomy and the animals allowed to recover.

The corpora lutea were excised from the remainder of the ovary and the tissues weighed and fixed in Bouin's fixative as described previously.

#### 6.4.2. RESULTS.

The titres of steroid antibodies in jugular venous plasma from the control and testosterone-immunized ewes taken at the first laparotomy are shown in Table 6.6. Since the ewes were laparotomized 15-16 days after the previous experiment, the antibody titres were similar to those described earlier (section 6.3). High titres of testosterone antibodies (range 1:18000 - 1:30000) were present in plasma from the testosterone-immunized ewes. Whilst significant titres of androstenedione and oestradiol antibodies were found, a relatively low titre of oestrone antibodies was observed/

TABLE 6.6.

THE TITRE<sup>a</sup> OF STEROID ANTIBODIES IN TESTOSTERONE-IMMUNIZED EWES

<u>CONTROL</u>	<u><sup>3</sup>H-TESTOSTERONE</u>	<u><sup>3</sup>H-ANDROSTENEDIONE</u>	<u><sup>3</sup>H-OESTRADIOL</u>	<u><sup>3</sup>H-OESTRONE</u>
	1:1	N.S.	N.S.	N.S.
<hr/>				
TESTOSTERONE- IMMUNIZED				
9R-443	1:18000	1:59	1:250	1:4
OR-550	1:20000	1:240	1:270	1:6
9R-451	1:19000	1:700	1:170	1:4
9R-397	1:21000	1:110	1:450	N.S.
9R-589	1:30000	1:560	1:390	1:13

<sup>a</sup>Titre = The initial dilution of plasma which bound 50% of 15-20pg of tritiated steroid.

N.S. = &lt;50% binding of the tritiated steroid at a dilution of 1:1.

observed (Table 6.6).

The number of large follicles ( $\geq 5$ mm diameter) in the testosterone-immunized ewes at the first and second laparotomies are shown in Tables 6.7 and 6.8 respectively. The number of follicles greater than 5mm in diameter was higher in the testosterone-immunized ewes compared to the control value (Tables 6.7 and 6.8). Furthermore, a number of follicles greater than 11mm diameter was present in four testosterone-immunized ewes. These large follicles were absent in one testosterone-immunized animal

(9R-397, Tables 6.7 and 6.8) although the number of follicles (5-11mm diameter) was greater than the control value. The presence of corpora albicantia in two testosterone-immunized animals (9R-443 and 9R-397) indicated that, at some time, ovulation had occurred, albeit without behavioural oestrus.

The weight of non-luteal ovarian tissue and corpora lutea in the four testosterone-immunized ewes at the second laparotomy are included in Table 6.8.

The weight of the ovaries after the removal of corpora lutea varied widely both between and within the testosterone-immunized ewes. The weight of at least one ovary within each testosterone-immunized ewe was significantly greater than the control value (Table 6.8). In two ewes (9R-397, 9R-589, Table 6.8), a marked dimorphism between the ovaries was apparent. Whereas the weight of one ovary was significantly greater than the control value, the contralateral ovary was indistinguishable, macroscopically, from the ovaries in the control animals.

The/



TABLE 6.7.

THE OVARY IN TESTOSTERONE-IMMUNIZED EWES.<sup>a</sup> -First Laparotomy. (Before the booster immunization)

			No. of follicles Diameter (mm)	
Day of the cycle			5 - 11	>11
<u>IMMUNIZED</u>				
OR-550	RO	NBO	3	2
	LO		1	0
9R-443	RO	1	2	0
	LO		1	1
9R-397	RO	NBO	5	0
	LO		0	0
9R-451	RO	NBO	3	3
	LO		1	4
9R-589	RO	NBO	1	3
	LO		0	1
<u>CONTROL</u>			1.0 $\pm$ 0.8	0
(M $\pm$ SD)			(no. per animal)	
n			4	

RO = right ovary. LO = left ovary. NBO = no  
behavioural oestrus.

<sup>a</sup> - ovaries left in situ after the experiment.

TABLE 6.8.

THE OVARY IN TESTOSTERONE-IMMUNIZED EWES -  
Second laparotomy - after the booster immunization.

Day of the cycle			Wt.C.L. (mg)	Wt.NLT (mg)	No. follicles Diameter (mm)	
					5-11	>11
<u>IMMUNIZED</u>						
OR-550	RO	NBO	-	3797*	0	2
	LO		-	2088*	2	1
9R-397	RO	NBO	-	2391*	3	0
	LO		-	946	3	0
9R-451	RO		556,392	5824*	8	2
	LO		519	18332*	0	3
9R-589	RO	NBO	-	9088*	1	4
	LO			993	1	0
<u>CONTROL</u>			496	826	1.0 $\pm$ 0.8	0
(M $\pm$ SD)			$\pm$ 126	$\pm$ 229	(no. per animal)	
n			6	8	4	

C.L. = Corpora lutea. NLT = non-luteal tissue - ovary  
after the removal of C.L. NBO = no behavioural oestrus.  
n = number of observations.

\* Value lies outside corresponding control mean  $\pm$  2 S.D.

The weight of the corpora lutea in the testosterone-immunized ewe (9R-451) was similar to the control value (Table 6.8). This finding indicated that, although this animal did not show behavioural oestrus, ovulation had taken place.

The binding of  $^3\text{H}$ -testosterone, determined by charcoal absorption, by a 1/200 dilution of follicular fluid from the testosterone-immunized ewes, together with the testosterone binding in the same dilution of peripheral plasma is shown in Table 6.9. The binding of testosterone in follicular fluid was similar to the binding in peripheral plasma. This finding suggests that steroid antibodies were also present in the follicular fluid.

The plasma concentrations of testosterone, androstenedione, oestradiol and oestrone in the peripheral and ovarian venous plasma from the testosterone-immunized ewes taken at the first and second laparotomies are shown in Tables 6.10 and 6.11 respectively.

The concentration of testosterone and androstenedione in peripheral and ovarian venous plasma from the testosterone-immunized ewes was significantly higher at both laparotomies than the corresponding control values (Tables 6.10 and 6.11). Similarly, the levels of oestradiol and, to a lesser extent, oestrone, in peripheral and ovarian venous plasma from the testosterone-immunized ewes were higher than the corresponding control values.

#### 6.5. DISCUSSION.

All five ewes immunized against T-3-BSA responded to the immunization/

TABLE 6.9

THE BINDING OF 15-25pg OF  $^3\text{H}$ -TESTOSTERONE BY A 1/200DILUTION OF FOLLICULAR FLUID AND PERIPHERAL PLASMAFROM THE TESTOSTERONE-IMMUNIZED EWES

(First Laparotomy)

	Follicle diameter(mm)	% $^3\text{H}$ -testos- terone bound		Follicle diameter(mm)	$^3\text{H}$ -testos- terone bound.
OR-550	16	85.7	9R-451	18	85.5
	14	86.8		16	90.1
	11	87.4		16	82.9
	11	89.6		13	85.9
	7	84.4		12	87.5
	Peripheral	86.0		12	86.1
9R-397	11	88.0		12	85.3
	10	88.5		Peripheral	75.7
	9	89.5	9R-589	20	86.7
	2x5	85.9		17	-
	Peripheral	92.1		14	86.5
9R-443	12	71.6		7	85.3
	9	79.6		Peripheral	86.0
	Peripheral	89.0			

TABLE 6.10.  
 THE CONCENTRATION OF OESTRADIOL, OESTRONE, ANDROSTENEDIONE AND TESTOSTERONE  
 IN PERIPHERAL AND OVARIAN VENOUS PLASMA FROM TESTOSTERONE-IMMUNIZED EWES  
 (First laparotomy - before the booster immunization)

	Oestradiol(ng/ml)		Oestrone(ng/ml)		Androstenedione(ng/ml)		Testosterone(ng/ml)	
	P	RO	P	RO	P	RO	P	RO
IMMUNIZED								
OR-550	0.071*	1.084*	0.052*	0.254*	21.6*	55.0*	3.2*	2.8*
9R-443	-	3.982*	0.056*	0.147	5.9*	16.9*	1.2*	1.2*
9R-397	0.084*	0.065	0.022	0.034	7.5*	7.5*	1.5*	1.5*
9R-451	0.073*	0.041	0.052*	0.046	41.1*	56.0*	8.0*	9.4*
9R-589	0.113*	9.216*	0.214*	1.468*	40.2*	90.9*	7.1*	10.2*
CONTROL	0.033	0.052	0.019	0.096	0.125	0.315	0.044	0.186
(M±SD)	+0.008	+0.032	+0.005	+0.043	+0.143	+0.151	+0.030	+0.132

P = peripheral. RO = right ovary. LO = left ovary.

\* Value lies outside corresponding control mean  $\pm$  2 S.D.

TABLE 6.11.  
 THE CONCENTRATION OF OESTRADIOL, OESTRONE, ANDROSTENEDIONE AND TESTOSTERONE  
 IN PERIPHERAL AND OVARIAN VENOUS PLASMA FROM TESTOSTERONE-IMMUNIZED EWES  
 (Second laparotomy - after the booster immunization)

	Oestradiol(ng/ml)			Oestrone(ng/ml)			Androstenedione(ng/ml)			Testosterone(ng/ml)			
	P	RO	LO	P	RO	LO	P	RO	LO	P	RO	LO	
<u>IMMUNIZED</u>													
	OR-550	0.043	0.038	1.05*	0.047*	0.141	0.159	9.2*	14.8*	19.1*	2.2*	3.1*	2.5*
	9R-397	0.082*	6.257*	0.996*	0.075*	0.205*	0.501*	6.9*	7.5*	59.6*	1.3*	1.5*	1.8*
	9R-451	0.250*	0.829*	0.326*	0.033*	0.083	0.132	143.1*	114.0*	132.3*	>6.0*	20.1*	23.3*
	9R-589	-	6.090*	3.614*	0.042*	0.712*	0.190*	137.6*	223.5*	181.5*	23.7*	26.2*	22.0*
<u>CONTROL</u> (M±SD)	0.033	0.052		0.019	0.096		0.125	0.315		0.044	0.186		
	+0.008	+0.032		+0.005	+0.043		+0.143	+0.151		+0.030	+0.132		

P = peripheral. RO = right ovary. LO = left ovary.

\* Value lies outside corresponding control mean  $\pm$  2 S.D.

immunization procedure as indicated by the presence of high titres of testosterone antibodies. The significant titres of oestradiol and androstenedione antibodies present in the testosterone-immunized ewes may have been due to the presence of antibodies directed towards structures common to both the steroid molecules and the testosterone-protein conjugate (the C-17 hydroxyl group in oestradiol molecule and the C4-5 double bond in the androstenedione molecule). Similarly, the low oestrone antibody titres may be attributed to the absence of a common functional group between oestrone and T-3-BSA.

The levels of LH both during anoestrus and during the breeding season in the testosterone-immunized ewes were similar to those found in the oestradiol-immunized animals. A comparison between the patterns of LH release during anoestrus in testosterone-immunized and oestradiol-immunized ewes is shown in figure 6.10. Although the frequency of spontaneous discharges of LH was somewhat higher in the testosterone-immunized ewes, the basal level of LH and the height of the LH peaks were similar in both groups (figure 6.10). It is likely, therefore, that the presence of oestradiol antibodies in the testosterone-immunized ewes had lowered the level of biologically active oestradiol, thereby reducing the negative feedback control of LH secretion. This hypothesis received support from the failure of exogenous oestradiol to influence the concentration of LH in the testosterone-immunized ewes suggesting that the biological activity of this steroid had been neutralized. A similar conclusion was drawn from the failure of exogenous oestradiol/

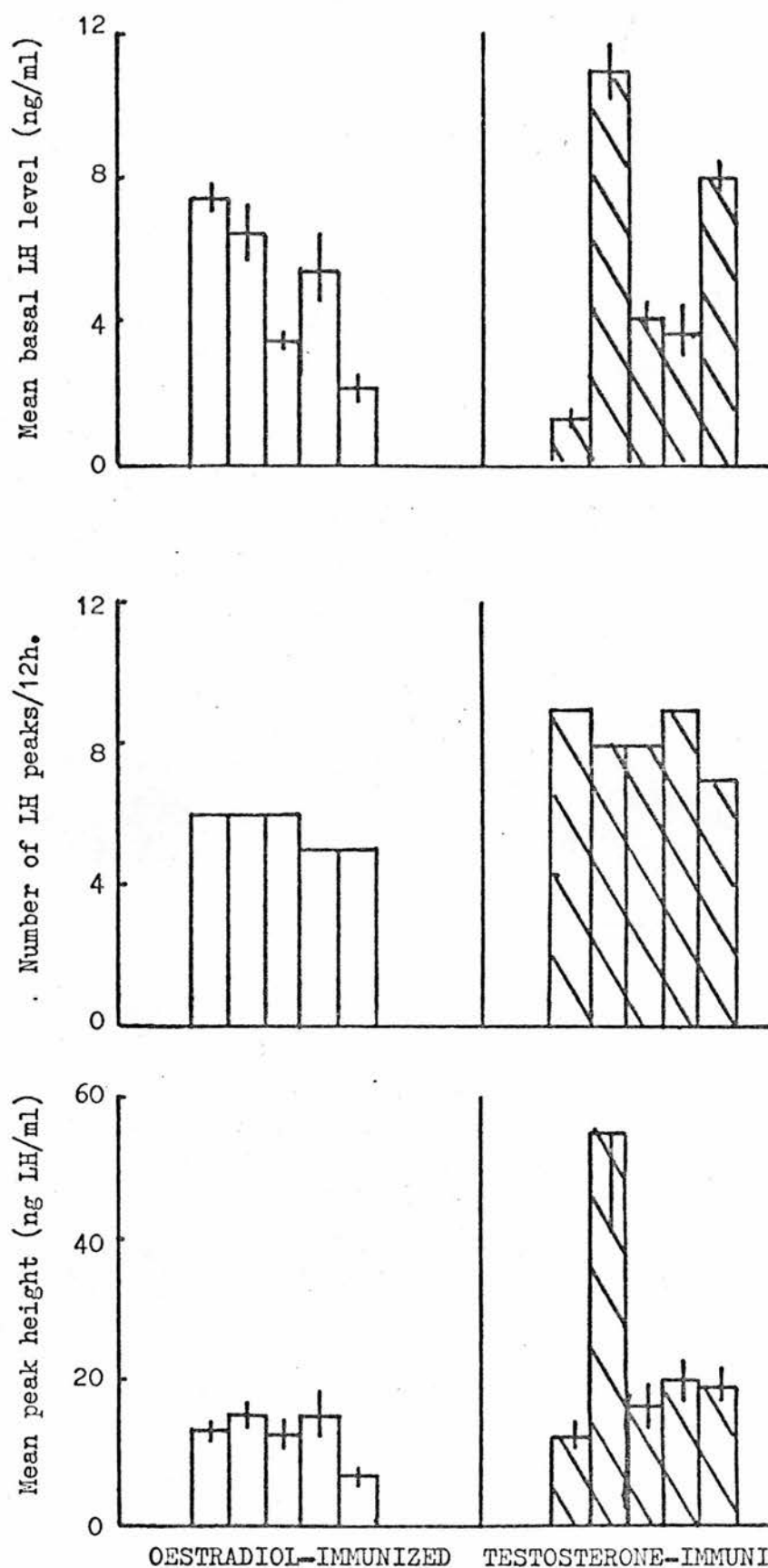


Figure 6.10 The pattern of LH release in oestradiol-immunized and testosterone-immunized ewes during anoestrus.

The values are arranged in order of decreasing oestradiol and testosterone antibody titres respectively.

The vertical line represents the range (mean  $\pm$  S.E.).



oestradiol to exert its positive and negative feedback actions on LH secretion in ovariectomized ewes actively immunized against oestradiol (Caldwell et al. 1970; Scaramuzzi et al. 1970a).

Perhaps the most significant finding in the present study was the absence of elevated FSH levels in the testosterone-immunized ewes both during anoestrus and during the breeding season. The basal level of LH in two testosterone-immunized ewes (9R-451, 9R-443, fig. 6.2) was equivalent to, or higher than, the basal level of LH in two oestradiol-immunized animals (OR-872, OR-535, fig. 5.4) which showed a significant elevation in the concentration of FSH. If the degree of elevation in the level of LH reflects the completeness of oestradiol neutralization, then an elevation in the concentration of FSH would have been expected in the two testosterone-immunized ewes.

The principal difference between the testosterone-immunized and oestradiol-immunized ewes lay in the titres of androstenedione and oestrone antibodies. It has been demonstrated that immunization against androstenedione produced a reduction in the plasma concentration of FSH (Chapter 4). If androgens in some way enhance the release of FSH, perhaps through an antagonism of the negative feedback action of oestradiol, then the presence of significant androstenedione antibody titres in the testosterone-immunized ewes (but not in the oestradiol-immunized animals) in conjunction with the high titres of testosterone antibodies may/

may have lowered the level of biologically active androgens and thus reduced the release of FSH (or increased the negative feedback action of oestradiol on FSH secretion). Since it is thought that FSH secretion is more sensitive to oestradiol than the release of LH, the net effect would have resulted in relatively normal levels of FSH.

There is some evidence in the rat to suggest that testosterone enhances the release of FSH. Pretreatment with testosterone (0.1-1.0mg) enhanced the increase of ovarian weight in immature female rats in response to HCG (Johnson & Neqvi, 1969a; Neqvi & Johnson, 1969). Since augmentation of the ovarian response to HCG by FSH forms the basis for bioassays of FSH (Steelman & Pohley, 1953; Igarishi & McCann, 1964), the enhanced ovarian response to HCG in rats pretreated with testosterone may be attributed to an increased release of endogenous FSH. This hypothesis received support from the finding that cyproterone acetate, an anti-androgen, or an antiserum to FSH antagonized the effects of testosterone (Johnson & Neqvi, 1969b). Furthermore, the effectiveness of testosterone to augment the ovarian response to HCG was inversely related to the dose of steroid (Johnson & Neqvi, 1969a). This finding suggests that whilst low doses of testosterone may enhance the release of FSH, high levels of this steroid suppress the release of FSH.

Testosterone (150 ng/ml) or dihydrotestosterone (150 ng/ml) enhanced the release of FSH from rat pituitaries in vitro without altering the release of LH (Mittler, 1972). More recently, Kao and Weisz (1975) showed that testosterone (0.1/

(0.1 - 1.0 ng/ml) increased the release of LH from the rat pituitary in vitro in response to stimulation with hypothalamic extracts. On the other hand, dihydrotestosterone enhanced the release of both LH and FSH (Kao & Weisz, 1975).

Conversely, it has been demonstrated that testosterone was ineffective in suppressing the level of FSH in castrated rats (Gay & Bogdanove, 1969), indeed, the pituitary FSH content was increased, suggesting that testosterone, in contrast to oestradiol, had stimulated the synthesis of FSH. Furthermore, under certain conditions, low doses of testosterone, ineffective in suppressing the gonadotrophin levels in orchidectomized male rats, could antagonize the negative feedback action of oestradiol (Gay & Dever, 1971).

It is perhaps somewhat hazardous to extrapolate the data obtained in rats and from in vitro studies to explain physiological events in sheep. Nevertheless, some evidence of enhanced follicular development was found in lactating (Restall et al. 1972) and anoestrous (Radford & Wallace, 1971) ewes treated with testosterone suggesting an increase in FSH secretion. It is possible, therefore, that androgens may play a role in the control of FSH secretion in the ewe.

The alterations in plasma gonadotrophin levels in the testosterone-immunized ewes described in the present study are in direct contrast to the effects of immunization against testosterone in the female rat where the level of FSH was elevated without alteration in the release of LH (Hillier et al. 1975a,b). The reason for this discrepancy is not clear. The rat differs from the human in that the release of/

of LH is more sensitive to oestradiol than the release of FSH (Gay & Dever, 1971; Mahesh et al. 1975). In this case, a reduction in the circulating level of biologically active oestradiol, either by the presence of oestradiol antibodies or a disruption of oestradiol synthesis, would have resulted in a preferential increase in FSH secretion.

It is not possible to state categorically that the testosterone-immunized ewes were anovulatory. Although the incidence of oestrus was reduced in these animals, presumably due to the neutralization of oestradiol, the presence of corpora albicantia and corpora lutea in three ewes indicated that ovulation had occurred in these animals without signs of behavioural oestrus. It is widely accepted that "silent" ovulation (i.e. ovulation without behavioural oestrus) may occur in ewes at the onset of the breeding season (Thorburn et al. 1969; Walton, Cunningham, Temple & Bowman, 1974) suggesting that behavioural oestrus is not an absolute prerequisite for ovulation.

The presence of large follicles in the ovaries from the testosterone-immunized ewes reflected the gonadotrophic stimulation caused by the elevated levels of LH, a situation similar to that found in the oestradiol-immunized animals. The reason for the apparent dimorphism in the ovarian response to the elevated gonadotrophin levels in two testosterone-immunized ewes is not clear.

The presence of elevated levels of testosterone, androstenedione, oestradiol and, to a lesser extent, oestrone in the peripheral plasma of the testosterone-immunized ewes indicated the presence of antibodies to these steroids.

The/

The concentration of androstenedione, and to a lesser extent, oestradiol in ovarian venous plasma was higher than the corresponding levels in peripheral plasma suggesting that the ovarian secretion of these steroids had been increased. It is interesting to note that the level of testosterone was similar in both peripheral and ovarian venous plasma. This apparent absence of increased testosterone secretion may have been due to the presence of steroid antibodies in the follicular fluid. The binding of steroids in follicular fluid was similar to the steroid binding in peripheral plasma suggesting that a proportion of the steroid antibodies were present in the follicular fluid. In this case, the presence of antibodies to androstenedione would have reduced the availability of androstenedione, a precursor of testosterone, thereby preventing the increased secretion of testosterone, and, to a lesser extent, oestradiol.

#### 6.6. CHAPTER SUMMARY.

1. The five ewes responded to active immunization against T-3-BSA with the production of antibodies to testosterone. Significant titres of androstenedione and oestradiol antibodies were also present due to antibodies directed towards structures common to both the steroid molecules and T-3-BSA.
2. A marked elevation in the concentration of LH during anoestrus and during the breeding season was present in the testosterone-immunized ewes. The similarity in the patterns of LH release during anoestrus in the testosterone-immunized and oestradiol-immunized animals together with the failure of/

of exogenous oestradiol to influence the secretion of LH in the testosterone-immunized ewes indicated that the presence of oestradiol antibodies in these animals had lowered the level of biologically active oestradiol thereby reducing the negative feedback control of LH secretion.

3. The presence of normal levels of FSH during anoestrus and during the breeding season in the testosterone-immunized sheep indicated a difference in the mechanisms in the control of LH and FSH secretion in the ewe. Some evidence has appeared in the literature to suggest that low levels of testosterone may enhance the release of FSH. In this case, the absence of circulating biologically active androgens in the testosterone-immunized ewes may have reduced the release of FSH thereby enhancing the negative feedback action of oestradiol.

4. Although the incidence of oestrus was reduced in the testosterone-immunized ewes it was not possible to deduce if the animals were in an anovulatory state.

5. The elevation of testosterone, androstenedione, oestradiol and, to a lesser extent, oestrone in the peripheral plasma from the testosterone-immunized ewes indicated that the clearance rate of these steroids from the circulation had been reduced, presumably by the presence of steroid antibodies.

6. Although ovarian steroid secretion rates could not be determined, the presence of a higher level of androstenedione and, to a lesser extent, oestradiol in ovarian venous plasma compared to the peripheral levels suggested that the ovarian secretion of these steroids had increased. In general, the concentration of testosterone was similar in both peripheral and/

and ovarian venous plasma. The apparent absence of an increased secretion of testosterone may have been due to a reduction in the availability of androstenedione, the precursor of testosterone by the presence of androstenedione antibodies in the follicular fluid.

7. The presence of overt follicular development, indicated by follicles greater than 11mm diameter, in the ovaries of the testosterone-immunized ewes reflected the elevated level of LH found in these animals. A dimorphism in the ovarian response to the elevated level of LH was found in two testosterone-immunized ewes. The reason for this dimorphic ovarian response remained obscure.

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CHAPTER 7.

THE CONCENTRATIONS OF GONADOTROPHINS AND OVARIAN STEROIDS

IN EWES ACTIVELY IMMUNIZED AGAINST

OESTRONE-6-(O-CARBOXYMETHYL)OXIME-BOVINE SERUM ALBUMIN.



### 7.1. INTRODUCTION.

Oestrone is secreted by the ovary in the non-pregnant ewe, although in smaller amounts than oestradiol (Short et al. 1963; Lindner et al. 1964; Baird et al. 1968b; McCracken et al. 1969; Moore et al. 1969). Little is known about the function of oestrone apart from its role as a precursor of oestradiol within the ovarian follicle (fig. 5.1).

It was suggested earlier in this thesis, in Chapter 4, that androstenedione could modulate the feedback actions of oestradiol on LH secretion through its extra-ovarian conversion to oestrone. If this hypothesis proves to be correct, then the neutralization of biologically active oestrone could well produce an alteration in the pattern of LH release similar to that found in the ewes immunized against androstenedione.

To test this hypothesis, five adult Welsh mountain ewes were actively immunized with 1.9 mg of oestrone-6-(O-carboxymethyl)oxime-bovine serum albumin ( $E_1$ -6-BSA) during November, 1974. Booster immunizations of 1.6 and 1.1 mg of antigen were given at 101 and 266 days respectively after the primary immunization.

The incidence of oestrus in the oestrone-immunized ewes following the primary immunization is shown in figure 7.1. In four oestrone-immunized ewes, oestrus occurred at regular intervals in synchrony with the control animals. The fifth ewe immunized against  $E_1$ -6-BSA (OR-662) showed two oestrous cycles, each 17 days in length, following the primary immunization, after which oestrus failed to occur during the remainder of the period (fig. 7.1).

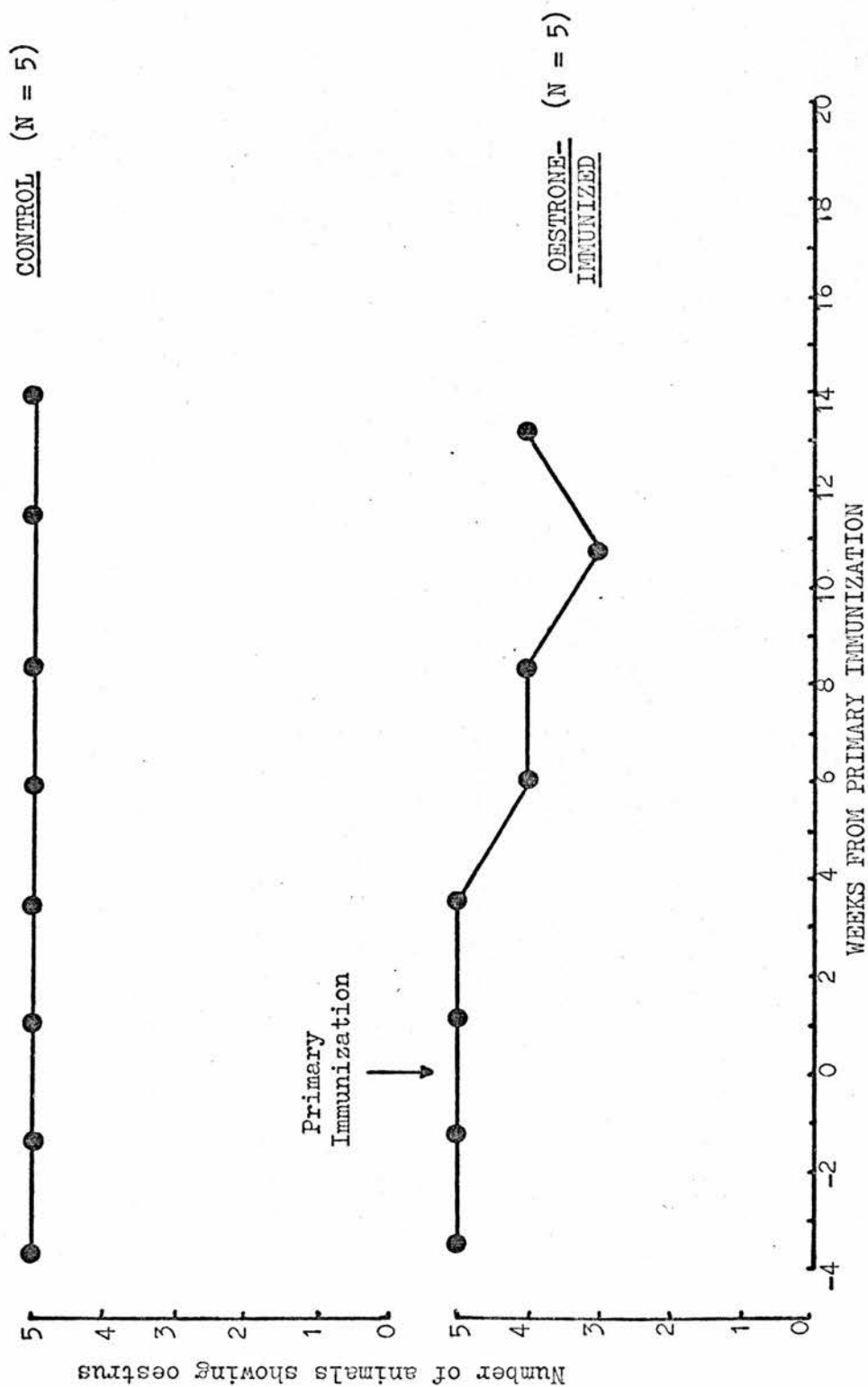


Figure 7.1. The incidence of oestrus in control and oestrone-immunized ewes following the primary immunization.

7.2. THE EFFECTS OF IMMUNIZATION AGAINST E<sub>1</sub>-6-BSA ON GONADOTROPHIN SECRETION DURING ANOESTRUS IN THE EWE.

7.2.1. PROCEDURE.

The present study was performed simultaneously with the anoestrus experiment described in the previous chapter.

During August 1975, 14 days after the oestrone-immunized ewes had received their second booster immunization, serial blood samples (5ml) were obtained from indwelling jugular vein cannulae from the five oestrone-immunized and four control animals. Samples were taken at 15-minute intervals for 12 hours (1300-0100h). Each oestrone-immunized ewe then received an intramuscular injection (at 0100h) of either 25 $\mu$ g or 50 $\mu$ g of OB (in 1ml of arachis oil) and sampling continued at 2-hourly intervals for 48 hours.

One week later, serial blood samples were again taken from the oestrone-immunized ewes at hourly intervals for 6 hours (2200-0100h). The ewes then received either 25 $\mu$ g or 50 $\mu$ g of OB (the opposite to the previous dose) at 0100h and samples were taken at 2-hourly intervals for 48 hours.

The level of LH was measured in all samples taken from each animal. The concentration of FSH was determined in alternate samples taken from each of three oestrone-immunized and three control animals.

The samples taken from each animal were pooled and the steroid antibody titres, together with the binding of steroids after equilibrium dialysis, measured in these pooled samples.

### 7.2.2. RESULTS.

The steroid antibody titres, determined by charcoal absorption, in plasma from the control and oestrone-immunized ewes are shown in Table 7.1. The titre of oestrone antibodies in plasma from the oestrone-immunized ewes was higher than the control value and ranged from 1:2700 to 1:13000. Significant titres of oestradiol (1:143 - 1:970) and testosterone (1:150 - 1:550) antibodies were also present in the oestrone-immunized animals. In contrast, although the binding of androstenedione in the oestrone-immunized ewes was higher than the control value, the titre of androstenedione antibodies (1:20 - 1:100) was lower than the other steroid antibody titres (Table 7.1).

The binding of all steroids, determined by equilibrium dialysis, in plasma from the oestrone-immunized ewes was significantly higher than the corresponding control values (Table 7.2).

The patterns of LH and FSH release during anoestrus in the control ewes have been described previously (Chapter 5, section 5.2). In brief, the basal level of LH was low (range: 0.23 - 0.64 ng/ml) with one spontaneous discharge of LH (range, peak heights: 2.67 - 5.35 ng/ml) present during the 12-hour period. The mean level of FSH ranged from 27.5 to 60.0 ng/ml.

The concentrations of LH in the five oestrone-immunized ewes during the 12-hour period before OB administration are illustrated in figures 7.2, 7.3 and 7.4. The frequency of spontaneous LH discharges during this period ranged from 7 to 8 peaks./

TABLE 7.1.  
THE TITRE<sup>a</sup> OF STEROID ANTIBODIES IN OESTRONE-IMMUNIZED EWES

CONTROL	<sup>3</sup> H-OESTRONE	<sup>3</sup> H-OESTRADIOL	<sup>3</sup> H-TESTOSTERONE	<sup>3</sup> H-ANDROSTENEDIONE
	N.S.	N.S.	1:1	N.S.
OESTRONE- IMMUNIZED.				
OR-428	1:2700	1:280	1:260	1:20
9R-652	1:9400	1:230	1:150	1:40
OR-566	1:13000	1:970	1:310	1:70
OR-662	1:7200	1:143	1:550	1:86
OR-801	1:11000	1:650	1:210	1:100

<sup>a</sup>Titre = The initial dilution of plasma which bound 50% of 15-20pg of tritiated steroid.

N.S. = <50% binding of the tritiated steroid at a dilution of 1:1.

**TABLE 7.2.**  
 THE PERCENTAGE (M $\pm$ S.E.) OF 15-25pg OF TRITIATED STEROID BOUND BY A 1/5 DILUTION OF PLASMA  
 FROM CONTROL AND OESTRONE-IMMUNIZED EWES DETERMINED BY EQUILIBRIUM DIALYSIS.

	<u><sup>3</sup>H-OESTRONE</u>	<u><sup>3</sup>H-OESTRADIOL</u>	<u><sup>3</sup>H-TESTOSTERONE</u>	<u><sup>3</sup>H-ANDROSTENEDIONE</u>
<u>CONTROL</u>	80.1 $\pm$ 3.1 (n=4)	88.4 $\pm$ 1.5 (n=4)	81.7 $\pm$ 1.1 (n=4)	57.2 $\pm$ 2.2 (n=4)
<u>OESTRONE- IMMUNIZED</u>	99.8 $\pm$ 0.0* (n=5)	99.5 $\pm$ 0.1* (n=5)	98.5 $\pm$ 0.3* (n=5)	97.8 $\pm$ 0.5* (n=5)

\* p<0.001. Immunized vs. Control (Student's t-test).

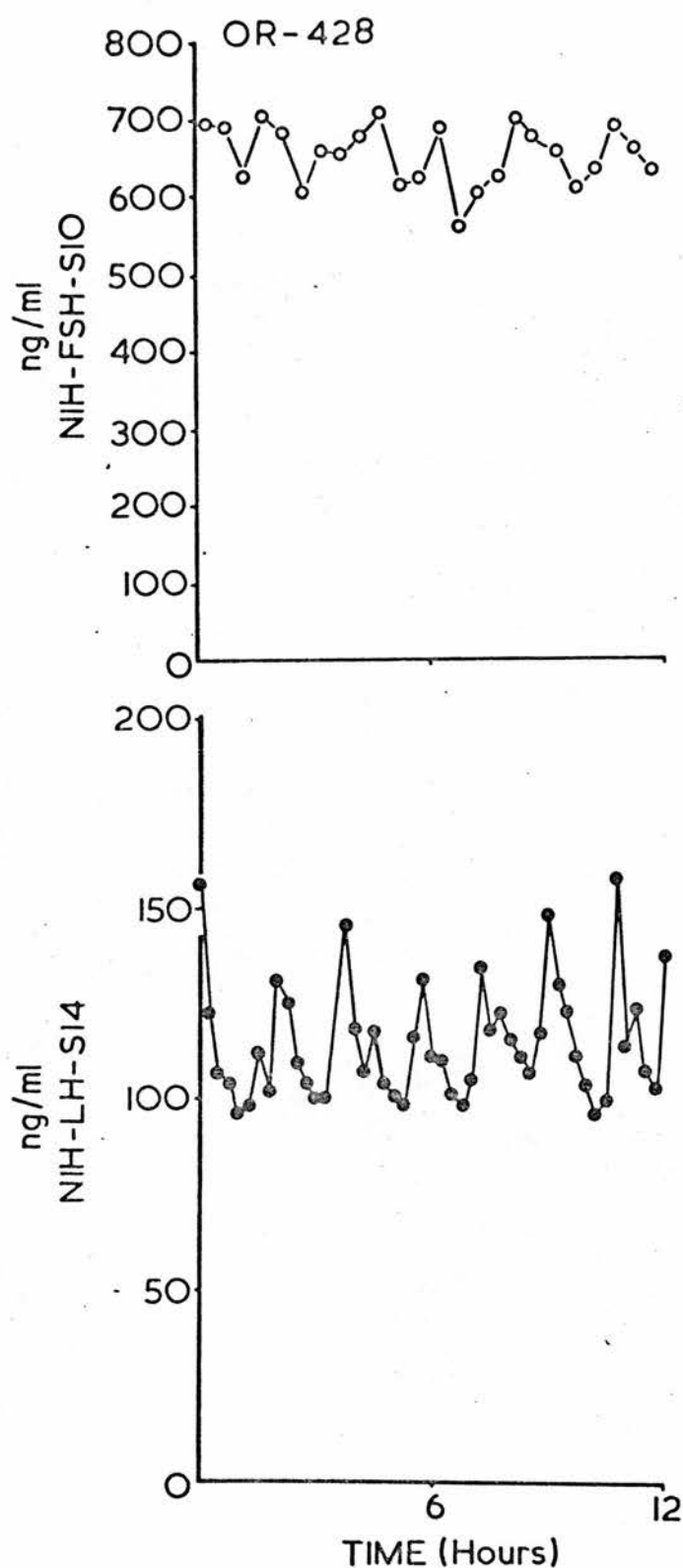


Figure 7.2. The plasma concentrations of LH and FSH during anoestrus in an oestrone-immunized ewe.

Samples were taken at 15-minute intervals for 12 hours. LH was assayed in every sample. FSH was measured in alternate samples.

OR-566

OR-662

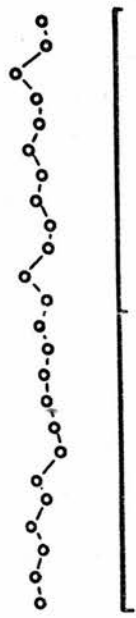
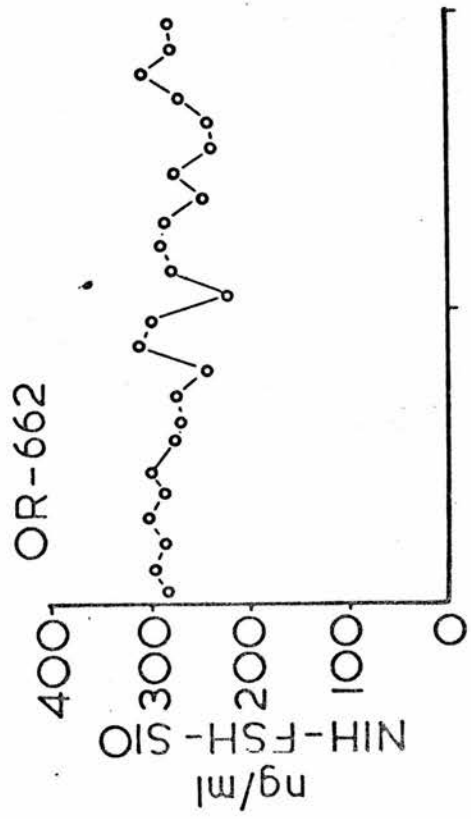


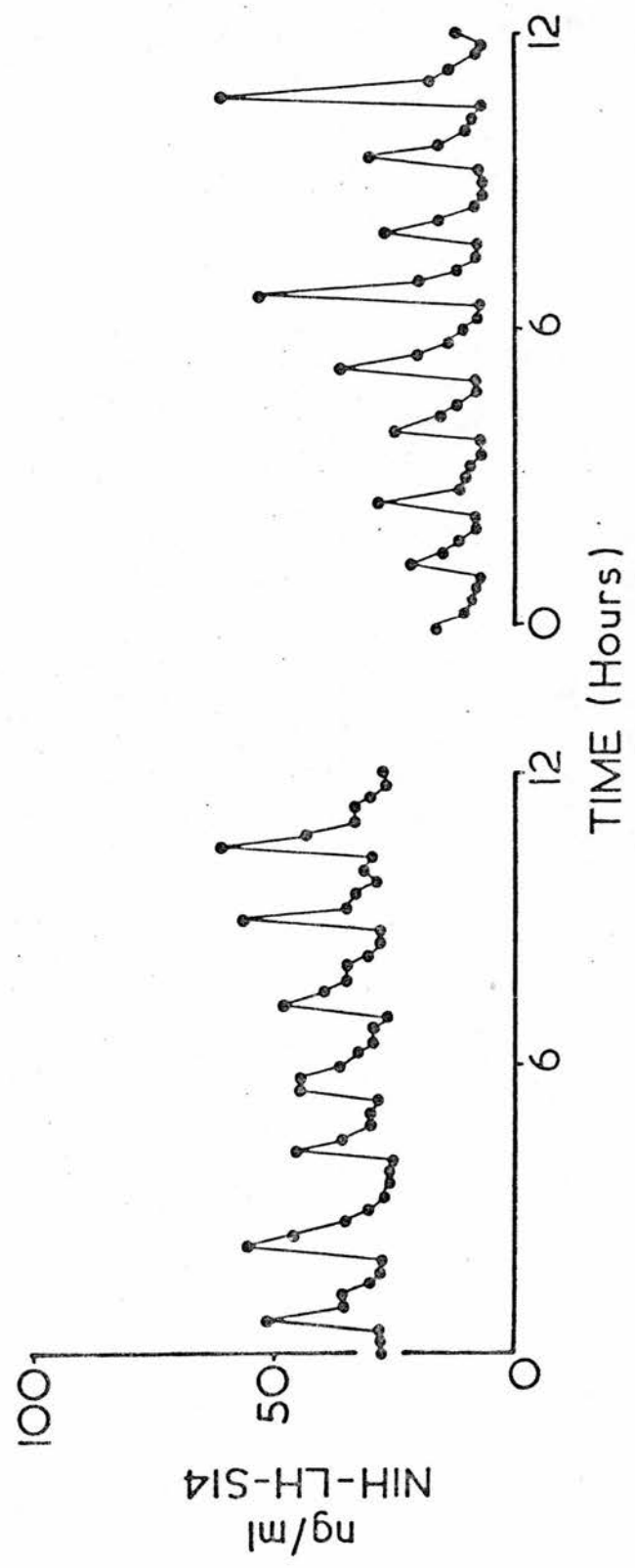
Figure 7.3.

The plasma levels of LH and FSH during anoestrus in two oestronone-immunized ewes.

Samples were taken at 15-minute intervals for 12 hours.

LH was assayed in every sample.

FSH was measured in alternate samples.





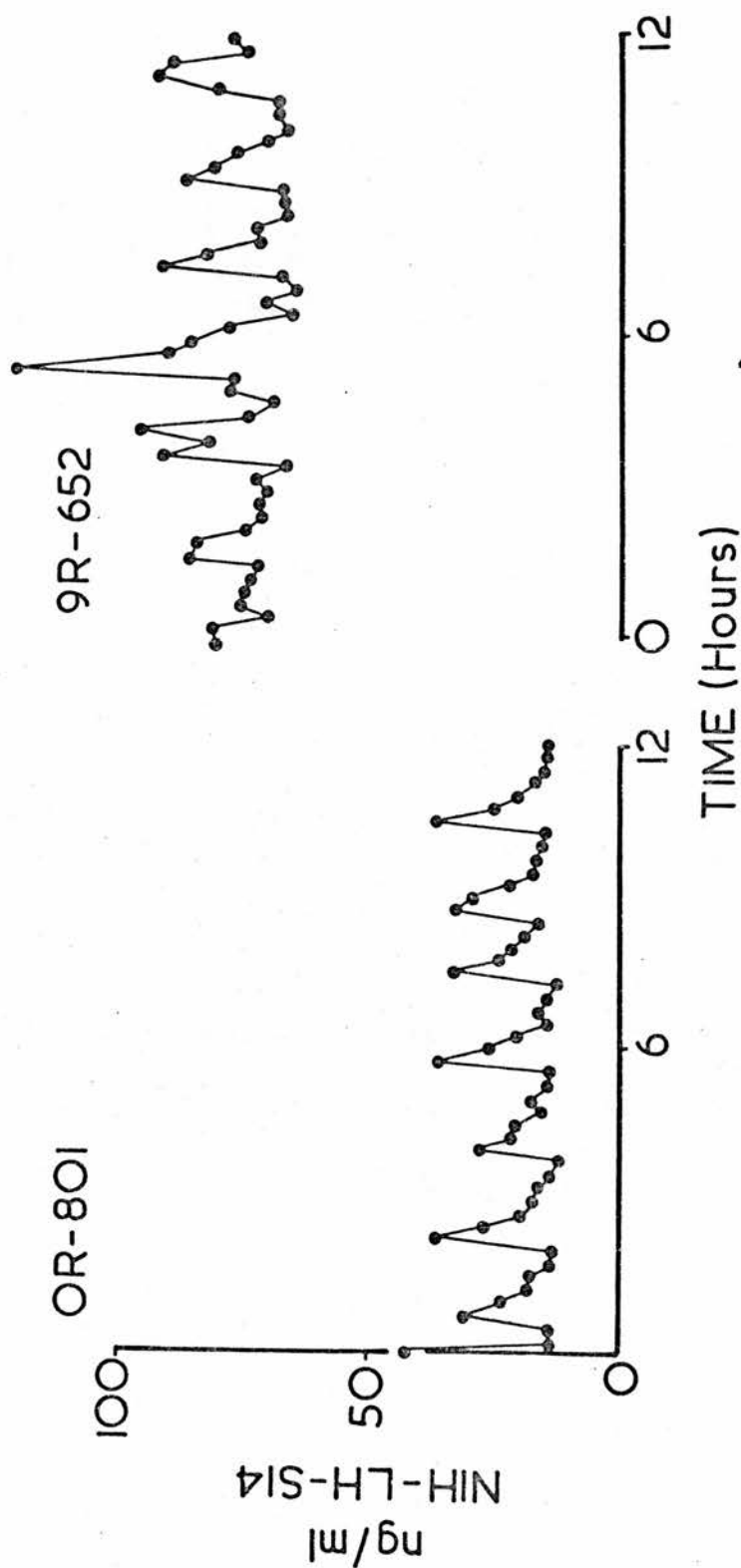


Figure 7.4. The plasma concentrations of LH during anoestrus in two oestrone-immunized ewes. Samples were taken at 15-minute intervals for 12 hours. LH was assayed in every sample.

peaks. The basal level of LH in the oestrone-immunized ewes ranged from 7.31 to 98.64 ng/ml and was significantly higher than the control value (fig. 7.5). The degree of elevation in the basal LH level, however, was not correlated with the oestrone antibody titre (fig. 7.5) since the ewe with the lowest titre (OR-428) showed by far the highest level of LH (fig. 7.5).

The concentration of FSH in peripheral plasma during the 12-hour period before OB administration in three oestrone-immunized ewes are also included in figures 7.2 and 7.3. A marked elevation in the concentration of FSH was apparent in two ewes (OR-428, fig. 7.2; OR-662, fig. 7.3). In the third ewe, (OR-566, fig. 7.3) the mean level of FSH, although greater than the individual control values, was not significantly different from the mean of the control group (fig. 7.6).

The basal level of LH and the mean concentration of FSH in the oestrone-immunized ewes following treatment with 25 $\mu$ g and 50 $\mu$ g of OB are shown in Table 7.3. Neither the mean concentration of FSH nor the basal level of LH following OB administration was significantly different from the corresponding pre-injection values (Table 7.3). No consistent elevation in the concentration of LH or FSH was apparent throughout the 48 hour period following OB administration. These findings suggest that neither the negative nor the positive feedback actions of oestradiol on gonadotrophin secretion were operative in the oestrone-immunized ewes.

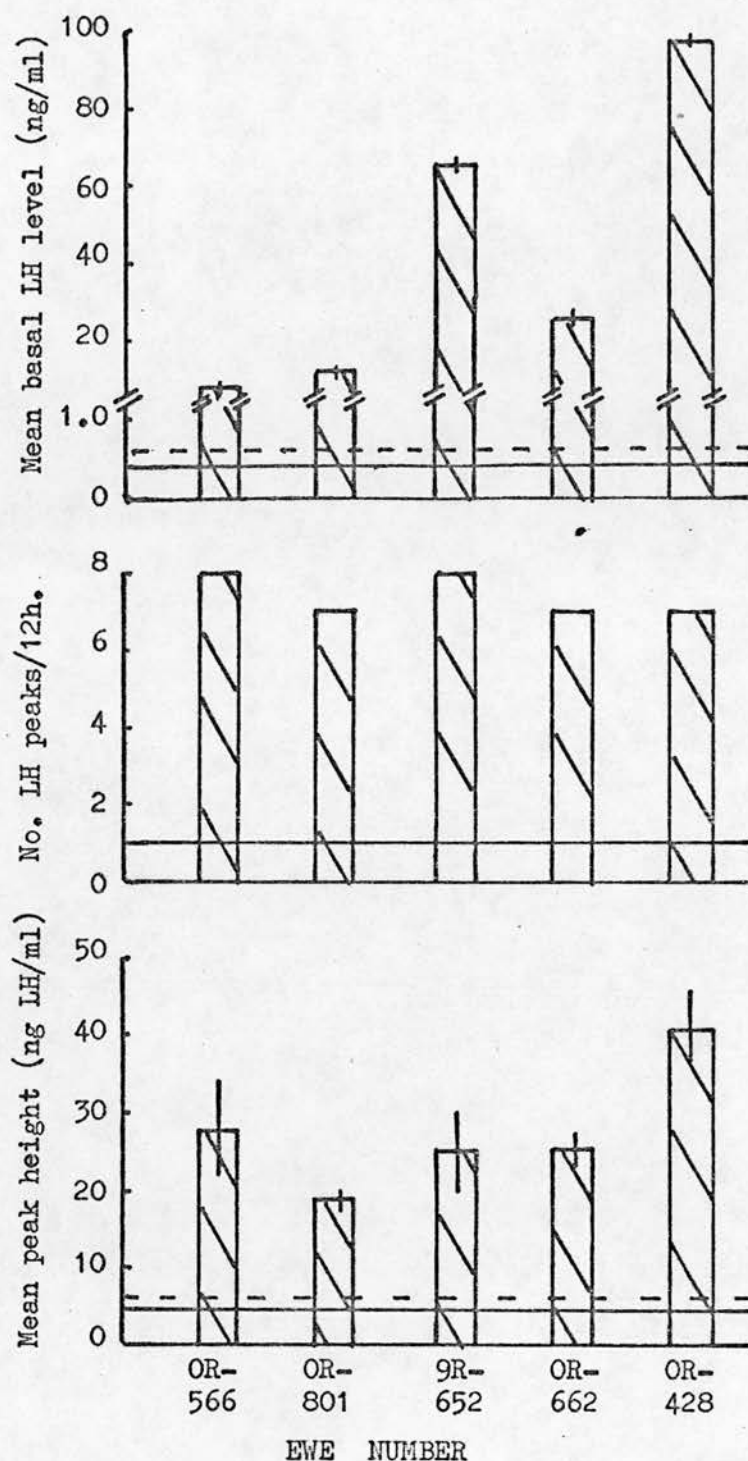


Figure 7.5. The pattern of LH release during anoestrus in oestrone-immunized ewes.

The values are arranged in order of decreasing oestrone antibody titre.

The vertical bars represent the mean  $\pm$  S.E.

The horizontal line represents the mean, and the broken line, the range (mean + 2S.D.) of the control group.

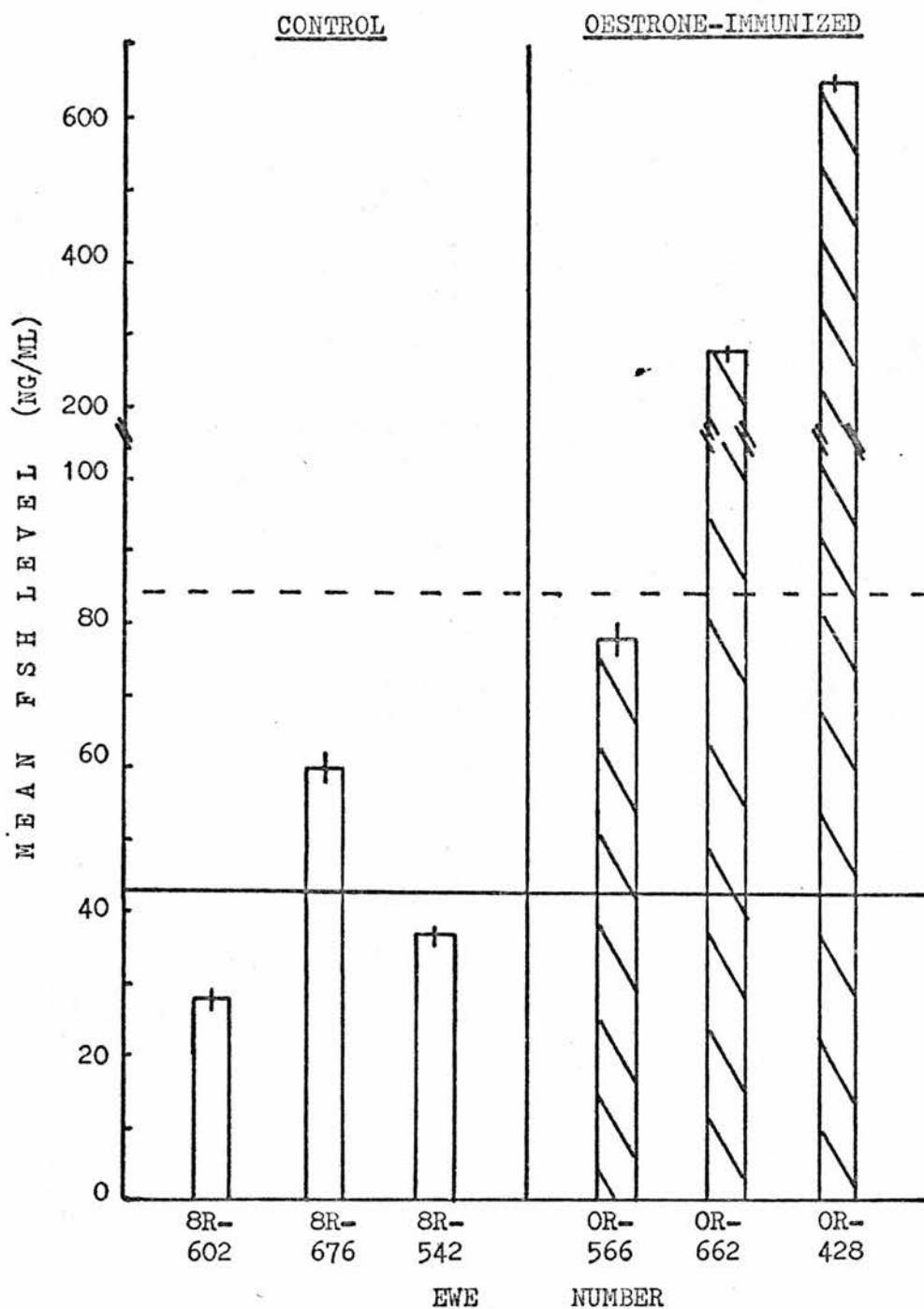


Figure 7.6. The mean plasma FSH concentration during anoestrus in control and oestrone-immunized ewes.

The oestrone-immunized values are arranged in order of decreasing oestrone antibody titre.

The vertical bars represent the mean  $\pm$  S.E.

The horizontal line represents the mean and the broken line, the range (mean + 2 S.D.) of the control group.

TABLE 7.3.  
THE MEAN CONCENTRATION OF FSH AND THE BASAL LEVEL  
OF LH IN OESTRONE-IMMUNIZED EWES TREATED  
WITH OESTRADIOL BENZOATE (OB)

	µgOB	LH (ng/ml; M±S.D.)		FSH (ng/ml; M±S.D.)	
		Pre-OB	Post-OB	Pre-OB	Post-OB
OR-566	25 <sup>a</sup>	7.31±0.36	4.56±0.79	77.4±9.3	80.0±11.2
	50 <sup>b</sup>		4.46±0.16		68.3± 6.9
OR-801	25 <sup>a</sup>	13.42±0.71	12.87±0.93	-	-
	50 <sup>b</sup>		10.34±0.72		-
9R-652	25 <sup>b</sup>	66.36±1.26	70.99±1.55	-	-
	50 <sup>a</sup>		65.30±2.42		-
OR-662	25 <sup>b</sup>	26.80±0.88	25.68±0.31	275.8±23.4	258.2±13.5
	50 <sup>a</sup>		26.44±0.26		257.0±24.1
OR-428	25 <sup>a</sup>	98.64±1.89	99.74±3.31	653.7±38.9	636.7±29.2
	50 <sup>b</sup>		82.99±3.94		613.1±26.5

Pre-OB - The pre-injection basal level of LH was determined by the mean of the 5 lowest concentrations of LH measured in samples taken at 15-minute intervals for 12 hours.

- The pre-injection level of FSH was determined by the mean of FSH levels measured in alternate samples.

Post-OB - The post-injection basal LH level was determined by the mean of the 5 lowest concentrations of LH measured in samples taken at 2-hourly intervals for 48 hours after steroid administration.

- The post-injection level of FSH was determined by the mean concentration of FSH measured in alternate samples.

<sup>a</sup> The steroid was administered immediately following the 12-hour sampling period.

<sup>b</sup> The steroid was administered one week after the previous experiment.

7.3. THE PLASMA CONCENTRATIONS OF LH, FSH AND PROGESTERONE  
DURING THE BREEDING SEASON IN THE OESTRONE-IMMUNIZED EWES.

7.3.1. PROCEDURE.

The present experiment was performed simultaneously with the studies on the gonadotrophin levels during the breeding season in oestradiol-immunized and testosterone-immunized ewes described in Chapters 5 and 6 respectively.

During September 1975, 50 days after their second booster immunization, the oestrone-immunized ewes received intravaginal progesterone sponges for the synchronization of oestrus. The sponges were removed after 12 days and, although the control ewes showed oestrus within 3-4 days, only one oestrone-immunized animal (OR-801) came into heat during this period and subsequently showed two oestrous cycles, each 16 days in length. Three of the four remaining oestrone-immunized ewes (OR-428, OR-566, OR-662) showed oestrus some 24 days following the termination of progesterone treatment. Of these three ewes, however, only one animal (OR-662) showed a second oestrus, 20 days later. The fifth oestrone-immunized ewe (OR-652) failed to show heat within the experimental period (i.e. within 40 days following the removal of the progesterone sponges). This animal, however, did eventually show oestrus for the first time 3 days after the completion of the present experiment.

Blood samples (5ml) were taken from the control ewes at 15-minute intervals for 6 hours on each of four days during the oestrous cycle (Table 7.4).

The/

TABLE 7.4.

THE SAMPLING PROTOCOL DURING THE BREEDING SEASON  
IN CONTROL AND OESTRONE-IMMUNIZED EWES

<u>CONTROL</u>	<u>Length of the cycle</u> <u>(days)</u>	<u>Sampling days<sup>a</sup></u>			
8R-602	17	-9	-5	-1	+1
8R-621	17	-9	-5	-1	+1
8R-676	16	-10	-6	-2	+1
8R-542	15		-7	-3	+1
<hr/>					
<u>OESTRONE-</u> <u>IMMUNIZED</u>					
OR-662	20	-13	-9	-5	+3
OR-801	16		-8		-4 +1
OR-652	NBO	"10"			
OR-566	NSO	+7	+11	+15	
OR-428	NSO	+8	+12	+16	

<sup>a</sup> Samples were taken at 15-minute intervals for 6 hours on each day. The sampling days were normalized around the day of the second oestrus or the days following oestrus.

NSO = No second oestrus.

NBO = No behavioural oestrus.

The four oestrone-immunized ewes which showed oestrus were also sampled at 15-minute intervals for 6 hours on each of 4 days following oestrus (Table 7.4). The fifth animal was sampled on day "10" of the cycle had she shown heat in synchrony with the control animals.

Where appropriate, the results were normalized either around the day of the second oestrus or the number of days following oestrus.

Plasma LH was assayed in all samples taken from each animal throughout the experiment. The concentration of FSH was determined in alternate samples taken from each of 3 oestrone-immunized and 3 control ewes.

The concentration of progesterone was measured in the first sample taken from each animal at each sampling period.

The steroid antibody titres were determined after pooling the samples taken from each animal.

### 7.3.2. RESULTS.

The steroid antibody titres, determined by charcoal absorption, in control and oestrone-immunized ewes are shown in Table 7.5. The titres were lower in the present experiment (Table 7.5) compared to the anoestrus study (Table 7.1) due to the longer interval after the booster immunization. Nevertheless, significant titres of oestrone (range: 1:1300 - 1:5600), oestradiol (range 1:32 - 1:420) and testosterone (range: 1:2 - 1:280) antibodies were present in the oestrone-immunized ewes.

The concentrations of LH, FSH and progesterone during the oestrous cycle in the control ewes has been described previously/



TABLE 7.5.  
THE TITRE<sup>a</sup> OF STEROID ANTIBODIES IN OESTRONE-INNUNIZED EWES

CONTROL	<sup>3</sup> H-OESTRONE	<sup>3</sup> H-OESTRADIOL	<sup>3</sup> H-TESTOSTERONE	<sup>3</sup> H-ANDROSTENEDIONE
	N.S.	N.S.	1:1	N.S.
<hr/>				
OESTRONE-INNUNIZED				
OR-428	1:1300	1:32	1:2	1:7
9R-652	1:2700	1:76	1:10	1:20
OR-566	1:2600	1:400	1:35	1:4
OR-662	1:5600	1:85	1:280	1:7
OR-801	1:4700	1:420	1:32	1:18

<sup>a</sup>Titre = The initial dilution of plasma which bound 50% of 15-20pg of tritiated steroid.

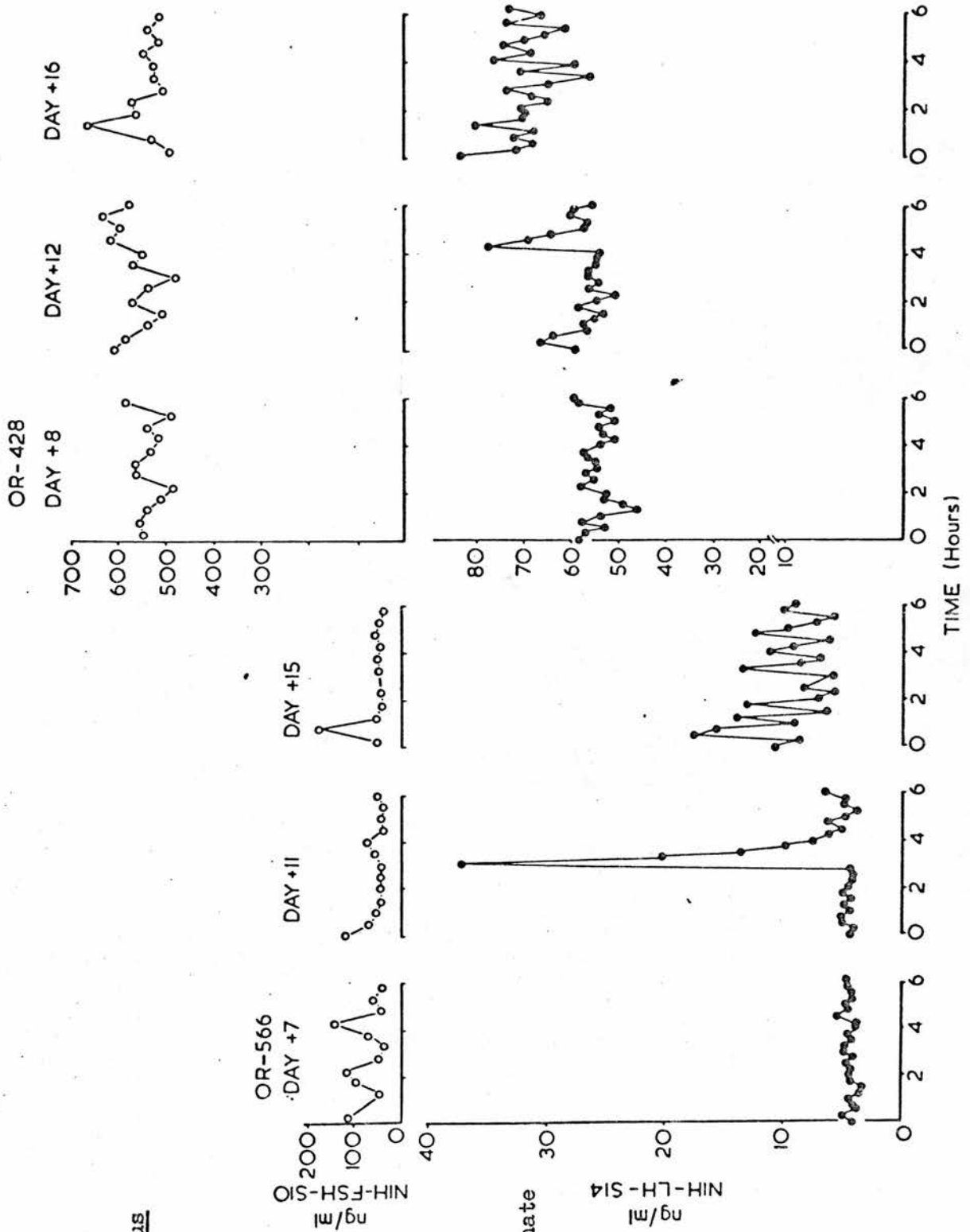
N.S. = <50% binding of the tritiated steroid at a dilution of 1:1.

previously (Chapter 5 section 5.3). In brief, maximal levels of progesterone were present between days -10 and -3 of the cycle. The level of progesterone then fell and low levels were present on day -1. The basal level of LH was low (range: 0.32 - 1.23 ng/ml) during the luteal phase of the cycle when high levels of progesterone were present. A rise in the basal level of LH was apparent during the follicular phase of the cycle, coincident with the low level of progesterone. The concentration of FSH remained relatively constant throughout the non-ovulatory period of the cycle although there was a tendency towards a lower level of FSH during the follicular phase compared to the luteal phase in two control animals.

The plasma concentrations of LH and FSH during the breeding season in the oestrone-immunized ewes are shown in figures 7.7, 7.8 and 7.9. The irregular incidence of oestrus in the oestrone-immunized ewes made it difficult to ascertain at which stage of the cycle these animals were sampled, thus making a direct comparison between the oestrone-immunized and control animals untenable. Nevertheless, it is apparent that the plasma levels of LH in the oestrone-immunized ewes (figs. 7.7, 7.8 and 7.9) were higher than those found during the non-ovulatory period of the oestrous cycle in the control animals (Table 7.6).

The plasma concentration of FSH in two oestrone-immunized ewes (OR-428, fig. 7.7; OR-662, fig. 7.8) was higher than the levels of FSH in the control animals (Table 7.6). The concentration of FSH in the third ewe (OR-566, fig. 7.7), however, was similar to the control values (Table 7.6).

Since daily progesterone values were not obtained throughout/



**Figure 7.7.**

The plasma levels of LH and FSH during the oestrous cycle in two oestron-immunized ewes.

Samples were taken at 15-minute intervals for 6 hours at each of the sampling periods.

LH was assayed in every sample.

FSH was measured in alternate samples.

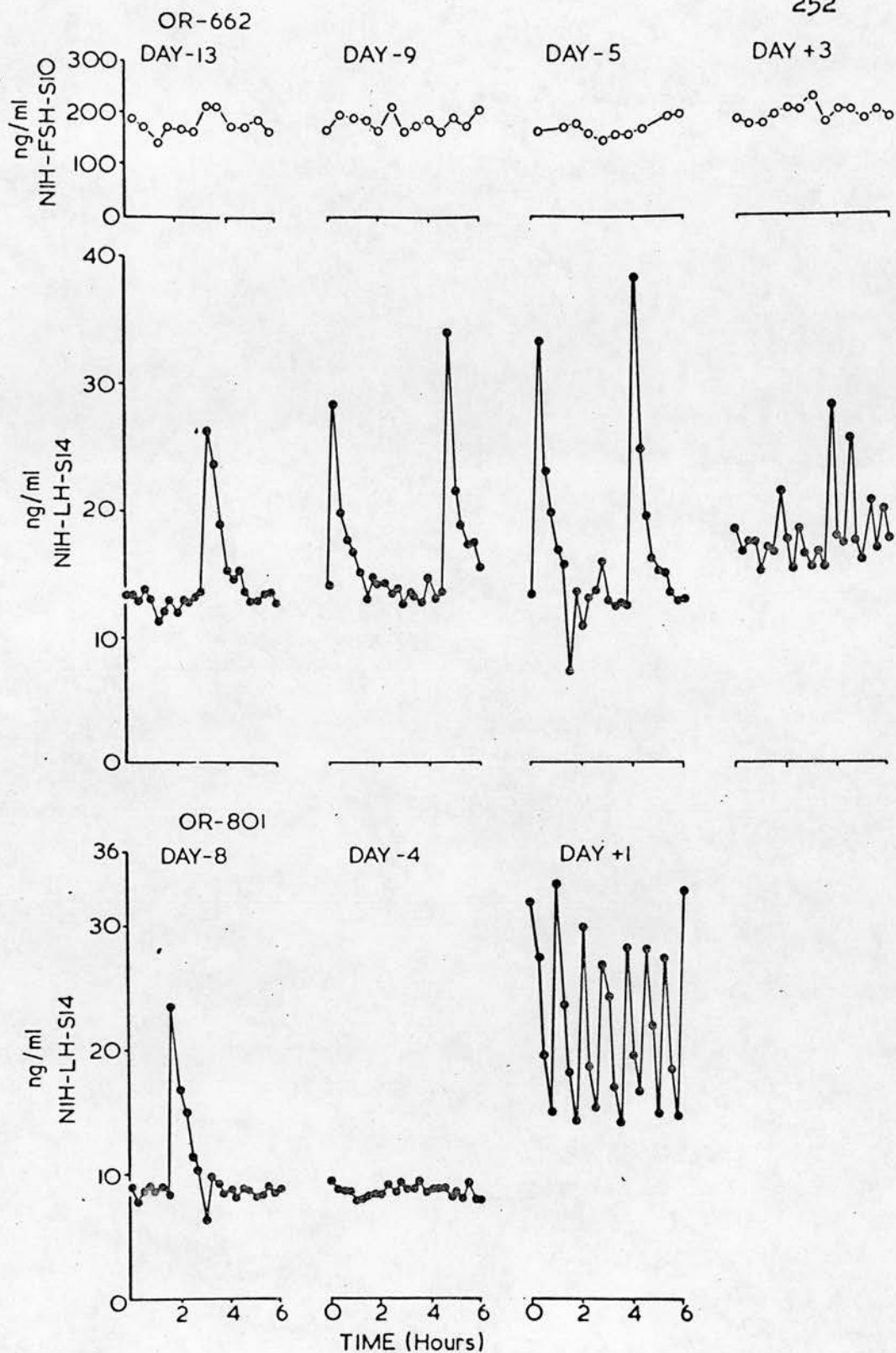


Figure 7.8. The plasma concentrations of LH and FSH in two oestrone-immunized ewes during the oestrous cycle.

Samples were taken at 15-minute intervals at each of the sampling periods. LH was assayed in every sample. FSH was measured in alternate samples from one animal.

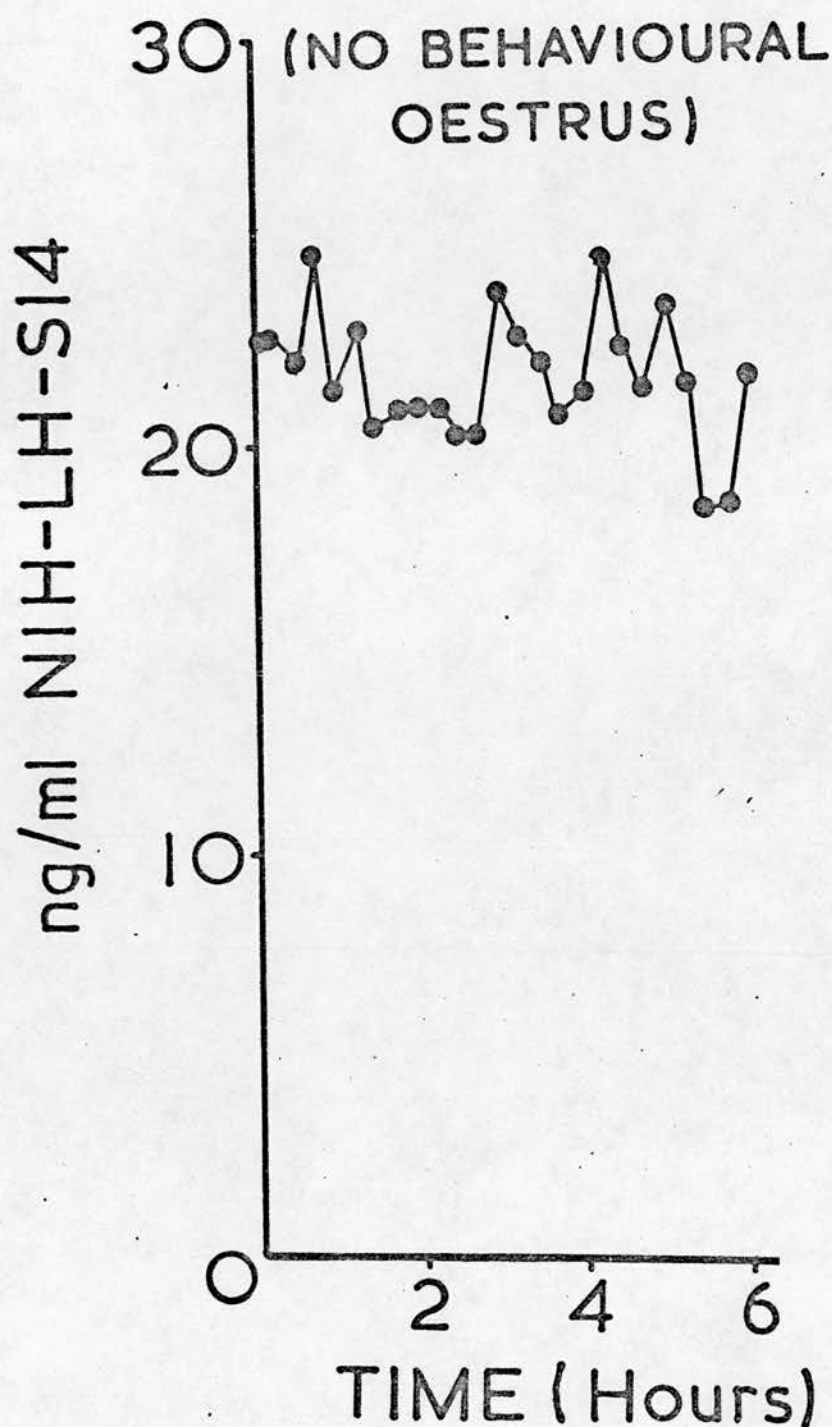


Figure 7.9. The plasma concentration of LH during the breeding season in an oestrone-immunized ewe.  
Samples were taken at 15-minute intervals for 6 hours. LH was assayed in every sample.

TABLE 7.6.

THE LEVELS OF LH, FSH AND PROGESTERONE DURING  
THE BREEDING SEASON IN CONTROL AND OESTRONE-IMMUNIZED EWES

	Days from oestrus	Mean FSH level (ng/ml)( $\pm$ S.E.)	Basal LH level (ng/ml)( $\pm$ S.D.)	Progesterone (ng/ml)( $\pm$ S.E.)
<u>CONTROL</u>	-10 to -9	75.3 $\pm$ 35.5(2)	0.73 $\pm$ 0.21(3)	1.6 $\pm$ 0.2(3)
	- 7 to -5	79.7 $\pm$ 25.9(3)	0.76 $\pm$ 0.21(4)	1.7 $\pm$ 0.1(4)
	- 3 to -2	79.5 $\pm$ 11.4(2)	0.74 $\pm$ 0.23(2)	1.3 $\pm$ 0.1(2)
	-1	32.7 (1)	1.56 $\pm$ 0.93(2)	0.2 (2)
	+1	74.3 $\pm$ 2.1(3)	1.11 $\pm$ 0.34(4)	0.2 $\pm$ 0.1(4)
<u>OESTRONE- IMMUNIZED</u>				
OR-662	-13	176.1 $\pm$ 5.6	12.06 $\pm$ 0.64	9.7
	- 9	181.5 $\pm$ 4.5	12.96 $\pm$ 0.34	8.4
	-5	167.3 $\pm$ 4.9	11.12 $\pm$ 2.32	7.8
	+3	191.2 $\pm$ 8.3	15.49 $\pm$ 0.41	1.6
OR-801	-8	-	7.65 $\pm$ 0.73	2.6
	-4	-	7.79 $\pm$ 0.10	1.4
	+1	-	14.49 $\pm$ 0.32	<0.1
9R-652	N.B.O.	-	19.49 $\pm$ 1.10	2.2
OR-566	+7	71.2 $\pm$ 13.2	3.65 $\pm$ 0.30	5.1
	+11	52.1 $\pm$ 5.9	4.10 $\pm$ 0.15	6.3
	+15	56.8 $\pm$ 11.3	5.71 $\pm$ 0.64	1.5
OR-428	+8	532.2 $\pm$ 8.7	49.61 $\pm$ 2.29	3.0
	+12	563.6 $\pm$ 11.9	53.59 $\pm$ 1.44	3.3
	+16	537.6 $\pm$ 13.5	62.26 $\pm$ 4.32	0.1

n = number of animals.

NBO = no behavioural oestrus.

throughout the experimental period, it was not possible to ascertain whether the oestrone-immunized ewes were undergoing normal oestrous cycles, albeit without the regular occurrence of behavioural oestrus. Luteal levels of progesterone were present in all the oestrone-immunized ewes on at least one sampling period during the present experiment. (Table 7.6.) In two ewes (OR-566 and OR-428, Table 7.6), the concentration of progesterone was lower at the final sampling period suggesting that luteal regression may have taken place.

#### 7.4. THE OVARIAN MORPHOLOGY AND STEROID CONCENTRATIONS IN THE OESTRONE-IMMUNIZED EWES.

##### 7.4.1. PROCEDURE.

Following the experiment during the breeding season described above, the oestrone-immunized ewes were laparotomized some 13-29 days later.

The laparotomy procedure followed the method described previously (Chapter 5, section 5.4). The ovaries were exposed and the number and diameter of corpora lutea and large follicles ( $\geq 5$ mm diameter) were recorded. A sample of blood was taken from the largest vein draining each ovary. Follicular fluid was aspirated from the largest follicles. A sample of jugular venous blood was obtained by venipuncture at the same time as the ovarian collections.

The ovaries and the adrenal glands were removed at the completion of the experiment. The corpora lutea were excised from the remainder of the ovary. The tissues were weighed and placed/

placed in the fixatives as described earlier.

The animals were killed with an overdose of anaesthetic at the completion of the experiment.

#### 7.4.2. RESULTS.

The titre of steroid antibodies in jugular venous plasma from the control and oestrone-immunized ewes at laparotomy are shown in Table 7.7. The titres were similar to those found in the previous experiment. Significant titres of oestrone (range: 1:900 - 1:4200), oestradiol (range: 1:12 - 350) and testosterone (range: 1:6 - 1:150) antibodies were present in the oestrone-immunized animals.

The weight of the corpora lutea and non-luteal ovarian tissue together with the number of large follicles ( $\geq 5$ mm diameter) in the oestrone-immunized ewes are shown in Table 7.8. Corpora lutea were present in 4 out of the 5 oestrone-immunized ewes even though one of these four animals (OR-566) had not shown behavioural oestrus for 27 days. The ovulation rate in these four animals ( $3.0 \pm 0.4$ ; mean  $\pm$  s.e.m.,  $n=5$ ) was significantly greater than the ovulation rate in the control ewes ( $1.5 \pm 0.5$ ; mean  $\pm$  s.e.m.,  $n=4$ ) ( $p < 0.05$ , Student's *t*-test). The weight of the non-luteal ovarian tissue in the four ovulatory oestrone-immunized ewes was similar to the control value (Table 7.8). The number of large follicles in these animals, however, was 2-4 times the number found in the control ewes, reflecting the increased ovulation rate.

The fifth oestrone-immunized ewe (OR-428, Table 7.8) failed to ovulate as shown by the absence of corpora lutea at laparotomy./



TABLE 7.7.  
THE TITRE<sup>a</sup> OF STEROID ANTIBODIES IN OESTRONE-IMMUNIZED EWES.

CONTROL	<sup>3</sup> H-OESTRONE	<sup>3</sup> H-OESTRADIOL	<sup>3</sup> H-TESTOSTERONE	<sup>3</sup> H-ANDROSTENEDIONE
	N.S.	N.S.	1:2	N.S.
OESTRONE- IMMUNIZED				
OR-428	1:900	1:12	1:6	1:4
9R-652	1:2000	1:74	1:7	1:3
OR-566	1:2400	1:300	1:15	1:6
OR-662	1:4000	1:50	1:150	1:5
OR-801	1:4200	1:350	1:13	1:2

<sup>a</sup>Titre = The initial dilution of plasma which bound 50% of 15-20pg of tritiated steroid.

N.S. = <50% binding of the tritiated steroid at a dilution of 1:1.

TABLE 7.8.

## THE OVARY IN OESTRONE-IMMUNIZED EWES

			Day of the cycle	Wt.C.L. (mg)	Wt.NLT (mg)	No. of follicles Diameter(mm)	
						5-11	> 11
<u>IMMUNIZED</u>							
OR-801	RO	9		804*	707	1	0
	LO			842*, 864*	759	0	0
OR-428	RO	NBO		-	3007*	5	0
	LO			-	2091*	6	0
OR-566	RO	NBO		-	942	2	0
	LO			531, 525, 646	666	2	0
OR-662	RO	7		388	1042	0	1
	LO			768, 397, 410	1551*	4	0
9R-652	RO	10		640	776	4	0
	LO			675	866	0	0
<u>CONTROL</u>				497	826	1.0±0.8	0
(M±SD)				126	229	(no. per animal)	
n.				6	8	4	

C.L. = corpora lutea. NLT = non-luteal tissue - ovary after the removal of C.L. RO. = right ovary. LO = left ovary.

n = number of observations. NBO = no behavioural oestrus.

\* Value lies outside the corresponding control mean  $\pm 2$  S.D.

laparotomy. The weight of non-luteal tissue in this animal was significantly greater than the control value. A large number of follicles ( $>5\text{mm}$  diameter) was present in this animal.

The weight of the adrenal glands in the oestrone-immunized ewes ( $2.28 \pm 0.4\text{g}$ ; mean  $\pm$  s.e.m.,  $n=10$ ) was significantly greater than the control value ( $1.71 \pm 0.30\text{g}$ ; mean  $\pm$  s.e.m.,  $n = 8$ ) ( $p < 0.01$ , Student's  $t$ -test).

The concentrations of oestrone, oestradiol, testosterone and androstenedione in peripheral and ovarian venous plasma from the oestrone-immunized ewes are shown in Table 7.9. The concentrations of oestrone and oestradiol in peripheral plasma from the oestrone-immunized ewes was significantly higher than the corresponding control values (Table 7.9). The concentrations of these steroids, in general was also elevated in the ovarian venous plasma from the oestrone immunized ewes.

The concentration of androstenedione in ovarian venous plasma from the oestrone-immunized ewes was significantly higher than the corresponding control value (Table 7.9). However, the level of this steroid in peripheral plasma was within the control range.

The concentration of testosterone in ovarian venous plasma from the oestrone-immunized ewes was similar to the control values (Table 7.9). The peripheral level of testosterone, however, was elevated in the two oestrone-immunized ewes with the highest testosterone antibody titres (OR-662, OR-566, Table 7.9).

#### 7.5. DISCUSSION.

**TABLE 7.9.**  
**THE CONCENTRATION OF OESTRADIOL, OESTRONE, ANDROSTENEDIONE AND TESTOSTERONE**  
**IN PERIPHERAL AND OVARIAN VENOUS PLASMA FROM OESTRONE-IMMUNIZED EWES**

	Oestradiol(ng/ml)			Oestrone(ng/ml)			Androstenedione(ng/ml)			Testosterone(ng/ml)		
	P	RO	LO	P	RO	LO	P	RO	LO	P	RO	LO
<u>IMMUNIZED</u>												
OR-801	0.069*	0.084	0.075	0.243*	0.260*	0.253*	0.067	1.126*	0.792*	0.098	0.093	0.307
OR-428	1.063*	0.229*	0.251*	2.122*	0.196*	0.238*	0.128	0.826*	1.472*	0.093	0.295	0.278
OR-566	0.086*	0.071	0.119*	3.048*	0.482*	0.414*	0.306	2.784*	12.980*	0.143*	0.274	0.314
OR-662	0.248*	0.532*	0.113	4.261*	0.706*	0.709*	0.183	7.993*	1.530*	0.203*	0.556	0.343
9R-652	0.040	0.050	0.028	0.429*	0.234*	0.179	0.210	0.808*	0.939*	0.076	0.271	0.262
<u>CONTROL</u>	0.033	0.052		0.019	0.096		0.125	0.315		0.044	0.186	
(M±SD)	±0.008	±0.032		0.005	±0.043		±0.143	±0.151		±0.030	±0.132	

P = peripheral. RO = right ovary. LO = left ovary.

\* Value lies outside corresponding control mean  $\pm$  2 S.D.

All five ewes immunized with E<sub>1</sub>-6-BSA responded to the immunization procedure as demonstrated by the presence of significant oestrone antibody titres. Significant, although comparatively low titres of oestradiol and testosterone antibodies were also present in plasma from the oestrone-immunized ewes.

The plasma concentrations of LH during anoestrus and during the breeding season in the oestrone-immunized animals were higher than the corresponding control values (fig. 7.5 and Table 7.6). The presence of antibodies to oestradiol in the oestrone-immunized ewes undoubtedly contributed towards the elevation in the plasma LH level, an observation consistent with the failure of exogenous oestradiol to exert its feedback actions on LH secretion (Table 7.3). The degree of alteration in the level of LH, however, was not positively correlated with the titre of oestrone or oestradiol antibodies, in fact, an inverse relationship between the oestrone antibody titre and the basal level of LH was apparent (fig. 7.5).

A comparison between the patterns of LH release during anoestrus in the oestrone-immunized ewes and the ovariectomized-hysterectomized animals described in Chapter 4 (Table 7.10) revealed a similar pulsatile pattern of release in both groups. Of greater interest, however, was the presence of basal levels of LH in four of the five oestrone-immunized ewes greater than the basal LH levels found in the ovariectomized-hysterectomized animals (Table 7.10). It is difficult to envisage a situation whereby LH levels are higher than those found in the absence of/

TABLE 7.10.  
THE PATTERNS OF LH AND FSH RELEASE DURING ANOESTRUS IN OESTRONE-IMMUNIZED AND  
OVARIECTOMIZED-HYSTERECTOMIZED EWES

	OESTRONE-IMMUNIZED					OVARIECTOMIZED-HYSTERECTOMIZED				
	OR-566	OR-801	9R-652	OR-662	OR-428	6R-125	6R-121	7R-227	7R-522	7R-465
<u>LH</u>										
Mean basal level <sup>a</sup> (ng/ml)	7.31	13.42	66.36	26.80	98.64	3.62	7.64	10.20	4.94	4.74
( $\pm$ SD) (n = 5)	$\pm 0.36$	$\pm 0.71$	$\pm 1.26$	$\pm 0.88$	$\pm 1.89$	$\pm 1.12$	$\pm 1.40$	$\pm 1.33$	$\pm 0.86$	$\pm 0.67$
Number of LH peaks/12 hours	8	7	8	7	7	10	9	10	13	10
Mean ( $\pm$ SE) peak height (ng/ml)	27.78	19.20	24.76	24.82	41.55	30.86	24.51	14.64	9.84	31.03
	$\pm 4.96$	$\pm 1.0$	$\pm 5.02$	$\pm 2.33$	$\pm 3.75$	$\pm 5.50$	$\pm 0.27$	$\pm 1.44$	$\pm 0.58$	$\pm 3.64$
<u>FSH</u>										
Mean concentration (ng/ml)	77.4	-	-	275.8	653.7	400.0	-	356.9	-	157.0
( $\pm$ SE) (n = 24)	$\pm 1.9$			$\pm 4.8$	$\pm 7.9$	$\pm 6.4$		$\pm 8.2$		$\pm 4.7$

n = number of determinations

<sup>a</sup> mean basal level = the mean of the 5 lowest concentrations of LH measured during the 12-hour period.

of all ovarian steroids unless some secretory product(s) from an organ apart from the ovary contributes in some way towards the maintenance of tonic LH secretion.

The adrenal glands are thought to be involved in the process of sexual maturation in the rat (Ramaley, 1972; Gorski & Lawton, 1973) and in humans (Collu & Ducharme, 1975). There is little evidence, however, to suggest that adrenal steroids play a role in the control of gonadotrophin secretion in the adult animal, although adrenal progesterone has been implicated in the control of the preovulatory LH surge in the rat (Feder, Brown-Grant & Corker, 1971; Lawton, 1973). In this context, it is interesting to note that in the non-pregnant ewe, oestrone is secreted in larger amounts by the adrenal glands in comparison to the ovaries (Baird et al. 1973). In the intact animal, active immunization with steroid-protein conjugates would result in the neutralization of both adrenal and ovarian steroid secretions. The significant increase in the adrenal weight in the oestrone-immunized ewes suggested that adrenal function may have been altered, although this observation awaits confirmation from the histological examination of the adrenals which was incomplete at the time of writing. Nevertheless, in the light of the very high plasma levels of LH found in the oestrone-immunized ewes, it is tempting to suggest that the neutralization of oestrone, arising from both the adrenals and the ovaries, in conjunction with a reduction in the level of biologically active oestradiol had produced LH levels greater than those found in the absence of all ovarian steroids. It would, however, be premature to postulate a role for adrenal oestrone in the control of LH secretion/

secretion in the ewe on the basis of the present results, indeed, the absence of a correlation between the titres of oestrone antibodies and the level of LH could suggest that alterations in the level of biologically active oestrone were unrelated to the alterations in the release of LH.

The plasma concentration of FSH was significantly elevated in two of the three oestrone-immunized ewes in which this hormone was measured. In these ewes (OR-662, OR-428) the concentration of FSH during anoestrus was similar to that found in the ovariectomized-hysterectomized animals (Table 7.10). The level of FSH in the third ewe (OR-566) lay within the control range during anoestrus (fig. 7.6) and during the breeding season (Table 7.6). This animal also showed the lowest plasma level of LH of the oestrone-immunized group. It is likely that the relatively low levels of gonadotrophins in this animal may have been due to the minimal alteration in the level of biologically active oestradiol since the concentrations of this steroid in peripheral and ovarian venous plasma were not significantly different from the corresponding control values (OR-566, Table 7.9).

A further anomalous finding observed in the present study was the morphological appearance of the ovaries in the oestrone-immunized ewes. In only two ewes (OR-428 and OR-662) was the weight of the non-luteal ovarian tissue greater than the control value (Table 7.8). There was little evidence of follicular stimulation since only one follicle greater than 11mm in diameter was found in the ovaries of the oestrone-immunized animals (Table 7.8). The morphological appearance of the ovaries in the oestrone-immunized ewes was in contrast to/



to the follicular ovaries found in the oestradiol-immunized and testosterone-immunized animals (Tables 5.8 and 6.7, 6.8). In the latter groups, in general, the ovaries were characterized by an increase in the weight of non-luteal ovarian tissue accompanied by multiple follicular development. These alterations were interpreted to result from the elevated plasma concentrations of gonadotrophins, an interpretation also proposed by Hillier et al. (1974, 1975a,b) and Ferin et al. (1974) for the presence of follicular ovaries in oestradiol-immunized rats and monkeys. It is surprising, therefore, that follicular ovaries were not found in the oestrone-immunized ewes since gonadotrophin levels in these animals were similar to, or greater than those found in the oestradiol-immunized and testosterone-immunized animals. The reason for this apparent lack of ovarian stimulation in the oestrone-immunized ewes is not clear. It is tempting to suggest that, in some way, the ovaries were refractory towards gonadotrophic stimulation. The presence of corpora lutea in four of the oestrone-immunized ewes, indicating that ovulation had taken place, indicated that the mature follicles were able to respond to the ovulatory stimulus.

In conclusion, active immunization against  $E_1$ -6-BSA produced several anomalous events which defy an adequate explanation at the present time. The study did little to confirm the hypothesis that androstenedione could modulate the feedback actions of oestradiol on LH secretion through its conversion to oestrone. The presence of high levels of LH together with the absence of ovarian stimulation in the oestrone/

oestrone-immunized ewes posed questions for which further studies are required before satisfactory answers can be found.

#### 7.6. CHAPTER SUMMARY.

- (1) All ewes immunized with  $E_1$ -6-BSA produced significant titres of oestrone antibodies.
- (2) The plasma concentrations of LH during anoestrus and during the breeding season were elevated in the oestrone-immunized ewes. Although the presence of oestradiol antibodies may have contributed towards the elevation in plasma LH, an observation consistent with the failure of exogenous oestradiol to exert its feedback actions on LH secretion, a satisfactory explanation for the presence of LH levels higher than those found in ovariectomized-hysterectomized animals could not be proposed on the basis of the present results.
- (3) The plasma concentrations of FSH during anoestrus and during the breeding season were elevated in two of the oestrone-immunized ewes. The level of FSH in the third ewe lay within the control range. It is possible that the alteration in the concentration of FSH may have been due to a reduction in level of biologically active oestradiol.
- (4) The morphological appearance of the ovaries in the oestrone-immunized ewes failed to reflect the elevated plasma levels of LH found in these animals. This finding was in contrast to the presence of follicular ovaries in the oestradiol-immunized and testosterone-immunized animals.
- (5) The occurrence of ovulation in four oestrone-immunized ewes indicated that the ovaries were responsive to gonadotrophin stimulation.
- (6)/

(6) Further studies are required to further examine the anomalous observations found in the present study on the effects of active immunization against E<sub>1</sub>-6-BSA in the ewe.

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CHAPTER 8GENERAL DISCUSSION AND CONCLUSIONS

Active immunization with steroid-protein conjugates provides, in theory, a technique whereby the biological actions of individual components of the gonadal (or adrenal) steroid spectrum may be neutralized. The involvement of a steroid in the feedback systems controlling the hypothalamic-hypophyseal-gonadal axis may be inferred if a reduction in the biologically active fraction of the steroid through antibody binding results in an alteration of gonadotrophin secretion.

The present studies examined the secretion of LH and FSH in non-pregnant ewes actively immunized against four steroids known to be secreted by the sheep ovary - oestradiol, androstenedione, oestrone and testosterone. Before discussing the possible physiological significance of these studies, it would be appropriate at this point to highlight some of the problems facing the interpretation of studies with active immunization against steroid-protein conjugates to delineate the feedback mechanisms controlling gonadotrophin secretion.

Two major problems confront the interpretation of active immunization studies. The first limitation lies in the evaluation of the specificity of the immune response in vivo. The second problem lies in the assessment of the degree to which the biological activity of the steroid is neutralized in vivo.

#### 8.1. THE SPECIFICITY OF THE IMMUNE RESPONSE IN VIVO.

In the present investigation, antibodies were detected by/

by an elevation in the binding of steroids by plasma after removal of the free steroid by dextran-charcoal absorption. Charcoal absorption, however, removed not only the free steroid but also steroid from the more labile steroid-antibody complexes (Collins, Barnard & Hennam, 1975; Odell, Silver & Grover, 1975). Since low affinity antibodies also contribute towards the in vivo plasma steroid binding, this method for determining plasma steroid binding cannot be directly equated to the true physiological situation. Nevertheless, some indication of high affinity steroid binding by antibodies may be gained with this technique.

The specificity of the immune response in the present studies was evaluated by a comparison of the steroid antibody titres defined as the dilution of plasma which bound 50% of the test steroids. It was not possible, using this technique, to ascertain whether the steroids were bound by specific antibodies or displaced other steroids bound by the same antibody.

The specificity of the immune response could be related to the functional groups present in both the antigen and the steroid under test. For example, the significant titre of oestradiol antibodies in the testosterone-immunized ewes (e.g. Table 6.1) might have arisen from the presence of antibodies directed towards the C-17 hydroxyl group common to both T-3-BSA (fig. 2.1) and oestradiol. In contrast, the absence of a significant oestradiol antibody titre in the androstenedione-immunized ewes (Table 4.1) probably reflected the absence of functional groups common to both A-11-BSA (fig. 2.1) and oestradiol.

Nieschlag/

Nieschlag (1975) suggested that a more realistic evaluation of the physiological situation may be gained from the determination of plasma steroid binding by equilibrium dialysis at 37°C. However, using this technique, a high percentage (50-89%) of the exogenous steroids were bound by plasma from the control ewes (e.g. Table 4.2). It was evident from a comparison of the percentage of steroid bound by control plasma measured by the two methods of equilibrium dialysis and charcoal absorption (fig. 4.1) that neither androstenedione nor the oestrogens were bound with a high degree of affinity, a finding also suggested by studies on the metabolism of oestrone and oestradiol in sheep (Kazama & Loncope, 1972; Challis, Harrison & Heap, 1973). A high proportion of the steroid bound by control plasma after equilibrium dialysis may be attributed to low affinity steroid-albumin interactions (Sandberg et al. 1957; Wu et al. 1976) and is still biologically active (Rosenfield, 1971; Vermeulen et al. 1971). Since an unknown proportion of steroid bound by plasma from the immunized ewes could also have been due to steroid-albumin complexes, the use of equilibrium dialysis was of limited value in the present studies.

The plasma steroid binding obtained by either charcoal absorption or equilibrium dialysis could not be directly related to physiological events in the immunized animals. One measurement which does bear some relationship to the physiological alteration in steroid binding is the presence of elevated steroid levels in the peripheral circulation. Active immunization against steroid-protein conjugates produced a marked elevation in the peripheral plasma concentration of the homologous/

homologous steroid (e.g. Nieschlag et al. 1974; Hillier et al. 1975a,b). It has been demonstrated that steroid antibodies delayed the clearance of steroids from the peripheral circulation (Schwartz et al. 1975; Wickings et al. 1976). This finding indicated that the affinity of the antibodies in vivo was sufficiently great to prevent, or reduce the peripheral metabolism of the steroid. Increased ovarian steroid secretion could also increase the concentration of steroid in the peripheral circulation. However, the peripheral concentration of androstenedione was not elevated in parallel with the level of this steroid in ovarian venous plasma from the oestradiol-immunized and oestrone-immunized ewes. This finding reflected the low androstenedione antibody titres found in these animals and suggested that steroid antibodies were the primary factor responsible for the elevation in concentration of steroids in peripheral plasma.

#### 8.2. THE DEGREE OF STEROID NEUTRALIZATION IN VIVO.

An equally important parameter together with specificity in the interpretation of immunological studies is the degree to which the biological activity of the steroid is neutralized through antibody binding.

Two major factors contribute towards the degree of steroid neutralization in the actively immunized animal - firstly, the binding capacity of the plasma and secondly, the relative affinities of the antibody and the target organ receptors for the circulating steroid.

The/



The binding capacity of the plasma is largely determined by the amount of antibody available to bind the circulating steroid. The compensatory steroid secretion in response to elevated gonadotrophin levels could lead to the saturation of the antibodies. This observation is particularly relevant to studies with long-term immunized animals (Thorneycroft et al. 1975) and can largely be overcome by frequent booster immunizations thereby increasing the antibody titre.

Equally important in the neutralization of steroids are the relative affinities of the antibodies and the steroid receptors within the target organs for the steroid. Unless the antibody affinity is greater than that of the steroid receptors, the circulating steroid could be bound by the receptors. This factor, coupled with the elevated steroid level would result in the hyper-stimulation of the target organs (Hillier & Cameron, 1975 a,b).

Insufficient time was available during the course of the present studies to determine the relative affinities of the antibodies and the hypothalamic steroid receptors. Nevertheless, a significant alteration in the plasma gonadotrophin concentration suggests that the affinity of the antibodies was sufficient to prevent the steroid from exerting its feedback action on gonadotrophin secretion.

The failure of steroid antibodies to alter gonadotrophin secretion could indicate two alternatives. Firstly, the titre and/or affinity of the antibodies was insufficient to significantly reduce the biologically active fraction of the steroid. Secondly, the steroid, although effectively bound by the/

the antibody, did not participate in the feedback control of gonadotrophin secretion.

In conclusion, it is apparent from the above discussion that some caution must be applied in the extrapolation of findings obtained through immunological studies to physiological events in the intact animal. Although both the specificity of the immune response and the degree of steroid neutralization within the actively immunized animal are difficult to assess using in vivo techniques, alterations in the gonadotrophin secretion produced by active immunization against steroid-protein conjugates may provide an insight into the feedback mechanisms controlling gonadotrophin secretion.

### 8.3. THE NEUTRALIZATION OF STEROID HORMONES IN THE EWE: AN INSIGHT INTO THE CONTROL OF GONADOTROPHIN SECRETION.

The classical techniques of ovariectomy and steroid replacement have contributed greatly towards the elucidation of the feedback mechanisms controlling gonadotrophin secretion. Whilst immunization with steroid-protein conjugates has been widely employed to produce antibodies for use in steroid radioimmunoassays, relatively little is known of the effects such hormonal disturbances have on the hypothalamic-hypophyseal-gonadal system within the immunized animal. The present studies describe the effects of active immunization against four steroids known to be secreted by the sheep ovary. Whilst some caution must be applied to the interpretation of these studies, the present investigation permits an insight into the mechanisms controlling gonadotrophin secretion in the non-pregnant/

pregnant ewe.

### 8.3.1. THE CONTROL OF LH SECRETION.

#### 8.3.1.1. The dual actions of oestradiol on LH secretion.

The classical endocrinological techniques of ovariectomy and steroid replacement have contributed towards the concept of a dual action of oestradiol in the control of LH secretion in the ewe (fig. 8.1).

Ovariectomy produced an elevation in the plasma concentration of LH characterized by a pulsatile pattern of release (figs. 4.7 and 4.8) (Butler et al. 1972; Reeves et al. 1972; Davis & Borger, 1974). Exogenous oestradiol suppressed LH secretion in the ovariectomized ewe (negative feedback) (figs. 4.7 and 4.8) and also induced a discharge of LH (positive feedback) some 19 hours after administration in both ovariectomized (figs. 4.7 and 4.8) (Scaramuzzi et al. 1970b, 1971) and intact anoestrous ewes (fig. 4.2) (Goding et al. 1972). A similar surge of LH may be induced by oestradiol treatment on day 3 of the oestrous cycle (Bolt et al. 1971). Furthermore, the ovarian secretion of oestradiol is increased on days 14-16 of the oestrous cycle, reaching maximal levels some 20-30 hours before the preovulatory LH surge (Moore et al. 1969; Scaramuzzi et al. 1970a; Cox et al. 1971; Bjersing et al. 1972; Baird et al. 1976a). The evidence described above serves to illustrate the intimate involvement of oestradiol in the control of LH secretion in the ewe.

The biological actions of oestradiol may be eliminated in the/

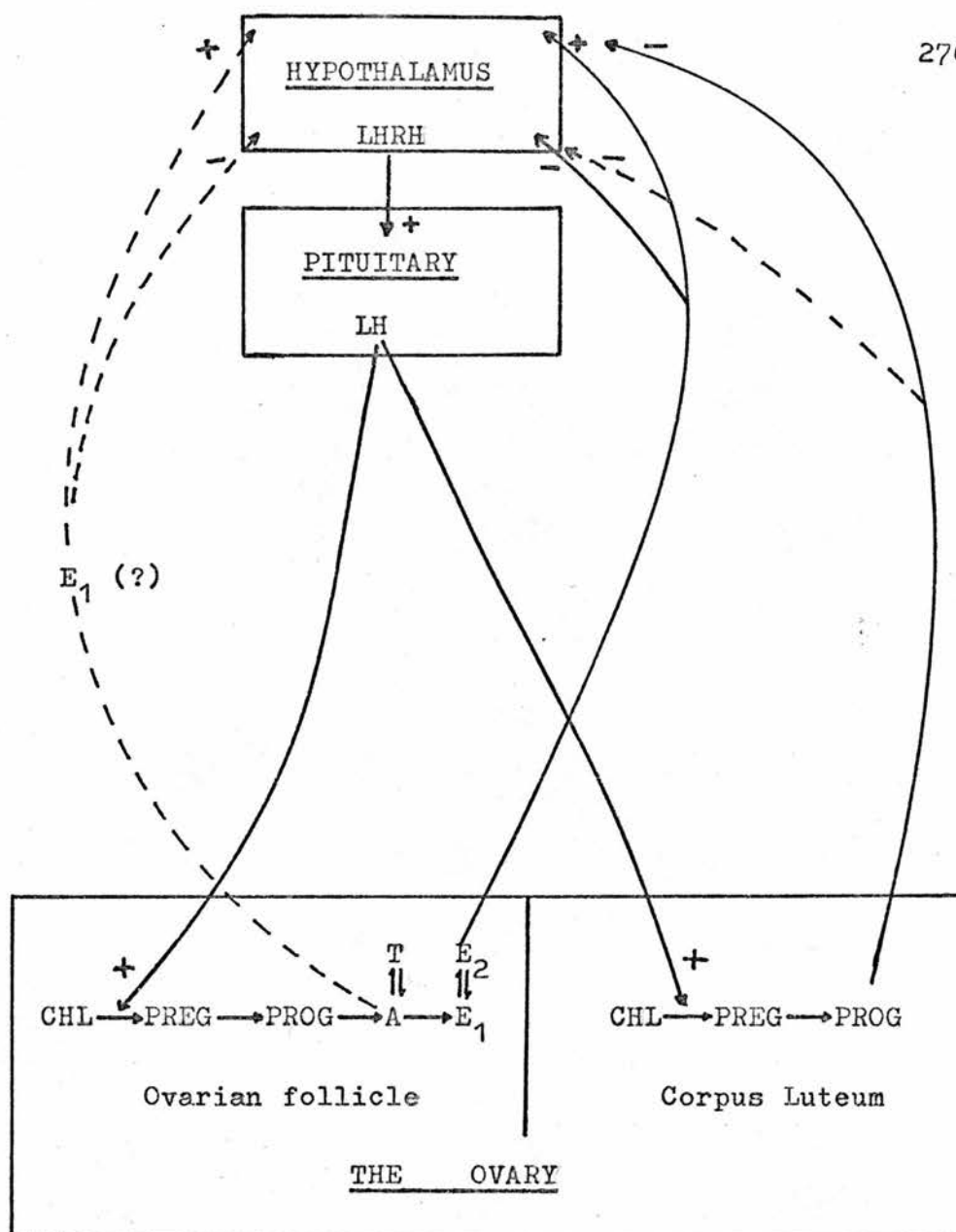


Figure 8.1.

THE CONTROL OF LH SECRETION IN THE EWE

The unbroken lines represent the accepted pathways of hormonal feedback control systems. The broken lines represent the postulated pathways of hormonal feedback action. (+) = positive action. (-) = inhibitory action.

CHL = Cholesterol; PREG = Pregnenolone; PROG = Progesterone;

A = Androstenedione; T = Testosterone; E<sub>1</sub> = Oestrone;

E<sub>2</sub> = Oestradiol-17 $\beta$ ; LH = Luteinizing hormone;

LHRH = Luteinizing hormone releasing hormone.

the intact animal by active or passive immunization against this steroid. Rats treated with an oestradiol antiserum on the day before proestrus failed to release the preovulatory surge of LH or ovulate (Ferin et al. 1969b). A similar blockade of ovulation was observed in ewes treated with an oestradiol antiserum on days 9-17 of the oestrous cycle (Fairclough et al. 1976a,b). These studies gave direct confirmation of the involvement of oestradiol in the ovulatory process in these species.

A generalized summary of the effects of active immunization against  $E_2$ -6-BSA (Chapter 5) is shown in Table 8.1. The plasma concentration of LH was significantly higher than the corresponding control values during anoestrus and during the breeding season. During anoestrus, the pattern of LH release in the oestradiol-immunized ewes was similar to that found in the ovariectomized-hysterectomized animals (Table 5.3) indicating that oestradiol is the major ovarian steroid with an inhibitory action on LH secretion.

The failure of four oestradiol-immunized ewes to show behavioural oestrus following progesterone treatment during the breeding season indicated a reduction in the availability of biologically active oestradiol. The ovaries from these four anoestrous oestradiol-immunized ewes showed multiple follicular development accompanied by an absence of corpora lutea (Table 5.8). The ovarian stimulation reflected the elevated plasma LH concentration in these animals (fig. 5.11). Since active immunization against oestradiol prevented exogenous oestradiol from exerting its positive feedback action on LH secretion in ovariectomized ewes (Caldwell et al. 1970/

A GENERALIZED SUMMARY OF THE EFFECTS OF IMMUNIZATIONAGAINST E<sub>2</sub>-6-BSA IN THE EWEAntibody titres

Oestradiol	- High
Oestrone	- High
Androstenedione	- Low
Testosterone	- Low

Anoestrus

Plasma LH level	- Elevated
Plasma FSH level	- 3/5 ewes - normal; 2/5 ewes - elevated

Breeding Season

Incidence of oestrus	- 1/5 ewes showed oestrus; 4/5 ewes - no oestrus
Plasma LH level	- Elevated
Plasma FSH level	- 3/5 ewes - normal; 2/5 ewes - elevated

Ovarian Morphology

Follicles	- 4/5 ewes - increased number of follicles > 11mm diameter
Ovulation rate	- 4/5 ewes - 0; 1/5 ewes - 3

Jugular venous steroid levels

Oestradiol	- Elevated
Oestrone	- Slightly elevated
Androstenedione	- Normal
Testosterone	- 2/4 ewes - normal; 2/4 ewes - slightly elevated

Ovarian venous steroid levels

Oestradiol	- Not measured
Oestrone	- Slightly elevated
Androstenedione	- Elevated
Testosterone	- 2/5 ewes - slightly elevated; 3/5 ewes - normal

1970; Scaramuzzi et al. 1970b), it is likely that in the intact, anoestrous oestradiol-immunized ewes, the circulating antibodies had prevented oestradiol from inducing the preovulatory LH surge and subsequent ovulation. A similar anovulatory condition accompanied by multiple follicular development and an elevated plasma LH concentration has been described in oestradiol-immunized rats (Hillier et al. 1974) and rhesus monkeys (Ferin et al. 1974).

The present study demonstrated that a reduction in the level of biologically active oestradiol through antibody binding produced an elevation in the plasma concentration of LH with a pattern of release during anoestrus similar to that found in ovariectomized-hysterectomized ewes. These findings confirm the effects of ovariectomy and steroid replacement on LH secretion and illustrate the major involvement of oestradiol in the feedback control of LH secretion.

The question arises as to whether other steroids secreted by the ovary are also involved in the control of LH secretion either directly, or through a modulation of the feedback actions of oestradiol. To examine this possibility, groups of ewes were actively immunized against androstenedione, oestrone and testosterone.

#### 8.3.1.2. A possible role for androstenedione in the control of LH secretion in the ewe.

Androstenedione is a major secretory product of the sheep ovary and is secreted quantitatively in larger amounts than oestradiol during anoestrus (Martensz et al. 1976) and during the/

the oestrous cycle (Baird et al. 1976a). Furthermore, a high correlation between the secretion of androstenedione and oestradiol is apparent during the oestrous cycle (Baird et al. 1976a) and both steroids are thought to arise primarily from the ovarian follicle (Baird & Scaramuzzi, 1976a). The close relationship between the ovarian secretion of androstenedione and oestradiol posed the question as to a possible role of androstenedione in the control of LH secretion in the ewe.

In an attempt to selectively neutralize the biological actions (if any) of androstenedione, a group of ewes was actively immunized against A-11-BSA (Chapter 4) (Table 8.2). The increased frequency of spontaneous LH discharges during anoestrus and the elevated basal level of LH during the luteal phase of the oestrous cycle in the androstenedione-immunized ewes (Table 8.2) suggested that in some way the inhibitory control of LH secretion had been reduced. The absence of a significant alteration in the binding of oestradiol by plasma from the androstenedione-immunized ewes determined by either charcoal absorption or equilibrium dialysis (e.g. Tables 4.4 and 4.5) suggested that the level of biologically active oestradiol had not been reduced through antibody binding. Since the concentration of oestradiol in ovarian venous plasma from the androstenedione-immunized animals did not increase in parallel with the level of this steroid in ovarian venous plasma (Table 4.10), the elevation in the plasma LH concentration may have arisen from a reduction in the secretion of oestradiol. However, behavioural oestrus, an oestradiol-dependent phenomenon (Robinson, 1959; Robertson, 1969) occurred at normal intervals during the breeding season in the androstenedione-immunized/



A GENERALIZED SUMMARY OF THE EFFECTS OF IMMUNIZATION  
AGAINST A-11-BSA IN THE EWE

Antibody titres

Androstenedione	- High
Oestradiol	- Very low
Oestrone	- Very low
Testosterone	- Low

Anoestrus

Frequency of LH discharges	- Increased
Plasma FSH levels	- Lower than controls
Effect of 25 µg of oestradiol benzoate	- Positive feedback delayed or absent

Breeding Season

Incidence of oestrus	- Regular, in synchrony with controls
Plasma LH level	- Elevated during luteal phase
Plasma FSH level	- Lower than controls

Ovarian Morphology

Follicles	- Double the number of follicles 5-11mm diameter
Ovulation rate	- 2

Jugular venous steroid levels

Androstenedione	- Elevated
Oestradiol	- Normal
Oestrone	- 2/4 ewes - normal; 2/4 ewes - slightly elevated
Testosterone	- 3/4 ewes - normal; 1/4 ewes - slightly elevated
Progesterone	- Elevated

Ovarian venous steroid levels

Androstenedione	- Elevated
Oestradiol	- Normal
Oestrone	- Normal
Testosterone	- Normal
Progesterone	- Normal for ovaries with corpora lutea

immunized animals. Indeed, no difference in the behavioural parameters of oestrus could be ascertained between the androstenedione-immunized and control ewes (I.J. Clarke and R.J. Scaramuzzi. Unpublished observations). It is likely, therefore, that the ovarian secretion of oestradiol occurred at normal levels during the oestrous cycle in the ewes immunized against androstenedione and could not account for the presence of elevated plasma concentrations of LH.

A modification in the positive feedback action of oestradiol was also apparent in the androstenedione-immunized ewes. The LH surge following treatment with oestradiol benzoate during anoestrus was delayed or absent in those animals with high titres of androstenedione antibodies (figs. 4.3 and 4.4). Furthermore, during the breeding season, the time of the preovulatory LH surge following the onset of behavioural oestrus (reflecting increased oestradiol secretion) was delayed by some 15 hours in the androstenedione-immunized ewes, (R.J. Scaramuzzi. Unpublished results). Conversely, anoestrous ewes bearing androstenedione implants produced a surge of LH in response to treatment with 5 $\mu$ g of oestradiol benzoate, a dose ineffective in inducing positive feedback in control animals (P.F.A. Van Look and R.J. Scaramuzzi. Unpublished results). The evidence described above suggests that androstenedione could modify the hypothalamic sensitivity towards oestradiol.

There is some evidence to suggest that androstenedione is metabolized to oestrone both in the peripheral circulation (Baird et al. 1968a) and within the central nervous system (Naftolin, Ryan, Davies, Reddy, Flores, Petro, Kuhn, White, Takaoka & White, 1975/

1975). It is possible, therefore, that the relatively large amounts of androstenedione secreted by the ovary during the course of a day could contribute a significant proportion of the total oestrogens in the ewe. In this context, it is conceivable that androstenedione, through its metabolite oestrone, could modulate the actions of oestradiol in the control of LH secretion in the ewe.

In conclusion, active immunization against A-11-BSA produced an elevation in the plasma concentration of LH. The specificity of the immune response determined by charcoal absorption, equilibrium dialysis and the peripheral steroid concentration, suggested that the antibodies were directed primarily against androstenedione and to a lesser extent, testosterone. Since negligible alteration in the binding of oestradiol, the major steroid involved in the control of LH secretion, could be ascertained, it is postulated that androstenedione, a steroid secreted in parallel with oestradiol by the ovary, may serve to modulate the feedback actions of oestradiol, perhaps through extra-ovarian conversion to oestrone (fig. 8.1).

#### 8.3.1.3. The effects of active immunization against $E_1$ -6-BSA on LH secretion in the ewe.

To examine further the hypothesis that androstenedione might modulate the actions of oestradiol on LH secretion through its extra-ovarian conversion to oestrone, an attempt was made to selectively neutralize the biological actions of oestrone through active immunization against  $E_1$ -6-BSA (Chapter 7) (Table/

A GENERALIZED SUMMARY OF THE EFFECTS OF IMMUNIZATION  
AGAINST E<sub>1</sub>-6-BSA IN THE EWE

Antibody titres

Oestrone	- High
Oestradiol	- High
Androstenedione	- Low
Testosterone	- Low

Anoestrus

Plasma LH level	- Elevated
Plasma FSH level	- 1/3 ewes - normal; 2/3 ewes - elevated
Effect of 25µg and 50µg of oestradiol benzoate	- No alteration in LH or FSH

Breeding Season

Incidence of oestrus	- Irregular
Plasma LH level	- Elevated
Plasma FSH level	- 1/3 ewes - normal; 2/3 ewes - elevated

Ovarian Morphology

Follicles	- Increased number of follicles 5-11mm diameter
Ovulation rate	- 1/5 ewes - 0; 4/5 ewes - 3-4

Jugular venous steroid levels

Oestrone	- Elevated
Oestradiol	- Elevated
Androstenedione	- Normal
Testosterone	- Normal

Ovarian venous steroid levels

Oestrone	- Elevated
Oestradiol	- Slightly elevated
Androstenedione	- Elevated
Testosterone	- Normal

(Table 8.3).

The studies on the oestrone-immunized ewes were somewhat confounded by the presence of significant titres of oestradiol antibodies (Table 7.1). A reduction in the level of biologically active oestradiol could have contributed towards the elevated plasma concentrations of LH found in the oestrone-immunized animals (figs. 7.2-7.4, 7.7-7.9). Furthermore, during anoestrus exogenous oestradiol failed to alter LH secretion in the oestrone-immunized ewes (Table 7.3).

The extent of the elevation in the plasma level of LH in the oestrone-immunized animals was such that the concentration of this hormone was, in general, higher than the levels of LH found in either the oestradiol-immunized or ovariectomized-hysterectomized ewes. The presence of LH levels greater than those found in the absence of all ovarian steroids suggested that an extra-ovarian steroid might play a role in the maintenance of tonic LH secretion. It is interesting to note that in sheep, more oestrone is secreted by the adrenal gland than by the ovary (Baird et al. 1973). Furthermore, the weight of the adrenal glands in the oestrone-immunized ewes was significantly greater than the control value. Whilst there is some evidence to suggest that adrenal steroids might be involved in sexual maturation in humans (Collu & Ducharme, 1975) and rats (Ramaley, 1972; Gorski & Lawton, 1972), it would be premature on the basis of the present study to postulate that adrenal oestrone exerts an inhibitory action on LH secretion in the adult ewe.

A further anomalous finding arose from the relatively normal appearance of the ovaries in the oestrone-immunized ewes (Table 7.8). Whilst multiple follicular development was evident in/

in the ovaries from the oestradiol-immunized (Table 5.8) and testosterone-immunized ewes (Tables 6.7 and 6.8) reflecting the elevated levels of LH in these animals, no such follicular development was evident in the oestrone-immunized animals (Table 7.8). Whilst it is tempting to suggest that the ovaries in the oestrone-immunized ewes were refractory towards the very high circulating levels of LH, the fact that ovulation had occurred, as indicated by the presence of corpora lutea, showed that mature follicles within the ovary were still responsive to the ovulatory gonadotrophin stimulus.

In conclusion, the study on the effects of active immunization against  $E_1$ -6-BSA yielded little information to confirm the hypothesis that androstenedione could modulate the actions of oestradiol on LH secretion through extra-ovarian conversion to oestrone. The underlying mechanism producing the very high levels of gonadotrophins together with the apparent ovarian refractoriness in the oestrone-immunized ewes remain to be clarified.

#### 8.3.1.4. The secretion of LH in ewes immunized against T-3-BSA.

Testosterone is also secreted by the ovary in sheep (Baird et al. 1968b, 1973; McCracken et al. 1969) although its pattern of secretion during the oestrous cycle remains to be clearly defined. Although the involvement of testosterone in the control of LH secretion in the male is well documented, the role (if any) of this steroid in the female remains obscure.

An attempt was made to selectively neutralize the biological actions of testosterone in the ewe by active immunization against T-3-BSA (Chapter 6) (Table 8.4).

A GENERALIZED SUMMARY OF THE EFFECTS OF IMMUNIZATION  
AGAINST T-3-BSA IN THE EWE

Antibody titres

Testosterone	- High
Androstenedione	- High
Oestradiol	- High
Oestrone	- Low

Anoestrus

Plasma LH level	- Elevated
Plasma FSH level	- Normal
Effect of 25 $\mu$ g and 50 $\mu$ g of oestradiol benzoate	- No alteration in LH or FSH

Breeding Season

Incidence of oestrus	- 4/5 ewes - no oestrus; 1/5 ewes - oestrus 43 days after progesterone
Plasma LH level	- Elevated
Plasma FSH level	- Normal

Ovarian Morphology

Follicles	- Increased number of follicles >11mm diameter
Ovulation rate	- 4/5 ewes - 0; 1/5 ewes - 3

Jugular venous steroid levels

Testosterone	- Elevated
Androstenedione	- Elevated
Oestradiol	- Elevated
Oestrone	- Slightly elevated

Ovarian venous steroid levels

Testosterone	- Elevated
Androstenedione	- Elevated
Oestradiol	- Elevated
Oestrone	- Slightly elevated



The secretion of LH in testosterone-immunized ewes during anoestrus assumed a pattern of release similar to that found in the oestradiol-immunized animals (fig. 6.10). The presence of significant oestradiol antibodies in the testosterone-immunized ewes (Table 6.1) could have contributed towards the elevation of LH through a reduction in the level of biologically active oestradiol. This hypothesis was confirmed by the failure of exogenous oestradiol to exert its negative and positive feedback actions on LH secretion during anoestrus in the testosterone-immunized animals (Table 6.3). Furthermore, behavioural oestrus, an oestradiol-dependent phenomenon, failed to occur in the testosterone-immunized ewes following progesterone during the breeding season.

The present study, therefore failed to provide an accurate assessment of the role (if any) of testosterone in the control of LH secretion in the ewe due to a non-specific reduction in the level of biologically active oestradiol.

#### 8.3.1.5. The action of progesterone on LH secretion

The present discussion on the control of LH secretion in the ewe would not be complete without a brief comment on the possible role of progesterone.

The inhibitory effect of progesterone on the positive feedback action of oestradiol on LH secretion has been described (Goding et al. 1970; Scaramuzzi et al. 1971; Baird & Scaramuzzi, 1976b) thereby providing a physiological mechanism to prevent the premature release of a preovulatory surge of LH during the luteal phase of the oestrous cycle.

Recently/



Recently, it has been suggested that progesterone may have an inhibitory action on LH secretion (Karsch et al. 1976) since progesterone will inhibit the post-ovariectomy elevation of LH when administered immediately after ovariectomy (Foster & Karsch, 1976; Karsch et al. 1976). Furthermore, the level of LH rises towards the end of the oestrous cycle when the concentration of progesterone is reduced following luteal regression (Karsch et al. 1976), a situation also found in the control animals examined during the present investigation. However, the failure of progesterone to suppress the plasma concentration of LH in ovariectomized ewes (Goding et al. 1970; Scaramuzzi et al. 1971) necessitates the need for further studies to confirm an inhibitory action of progesterone in the maintenance of tonic LH secretion.

#### 8.3.2. THE CONTROL OF FSH SECRETION IN THE EWE.

Despite the considerable knowledge of the factors involved in the control of LH secretion, relatively little is known about the mechanisms controlling the release of FSH in the ewe, due largely to problems in the measurement of circulating levels of this hormone.

Although the release of LH and FSH is thought to be governed by a common hypothalamic releasing hormone (Schally et al. 1971), alterations in the plasma concentrations of LH and FSH during the oestrous cycle of the ewe do not occur simultaneously (Salamonsen et al. 1973). This finding was confirmed during the course of the present studies where no direct relationship between the occurrence of spontaneous discharges of LH and alterations in the plasma/

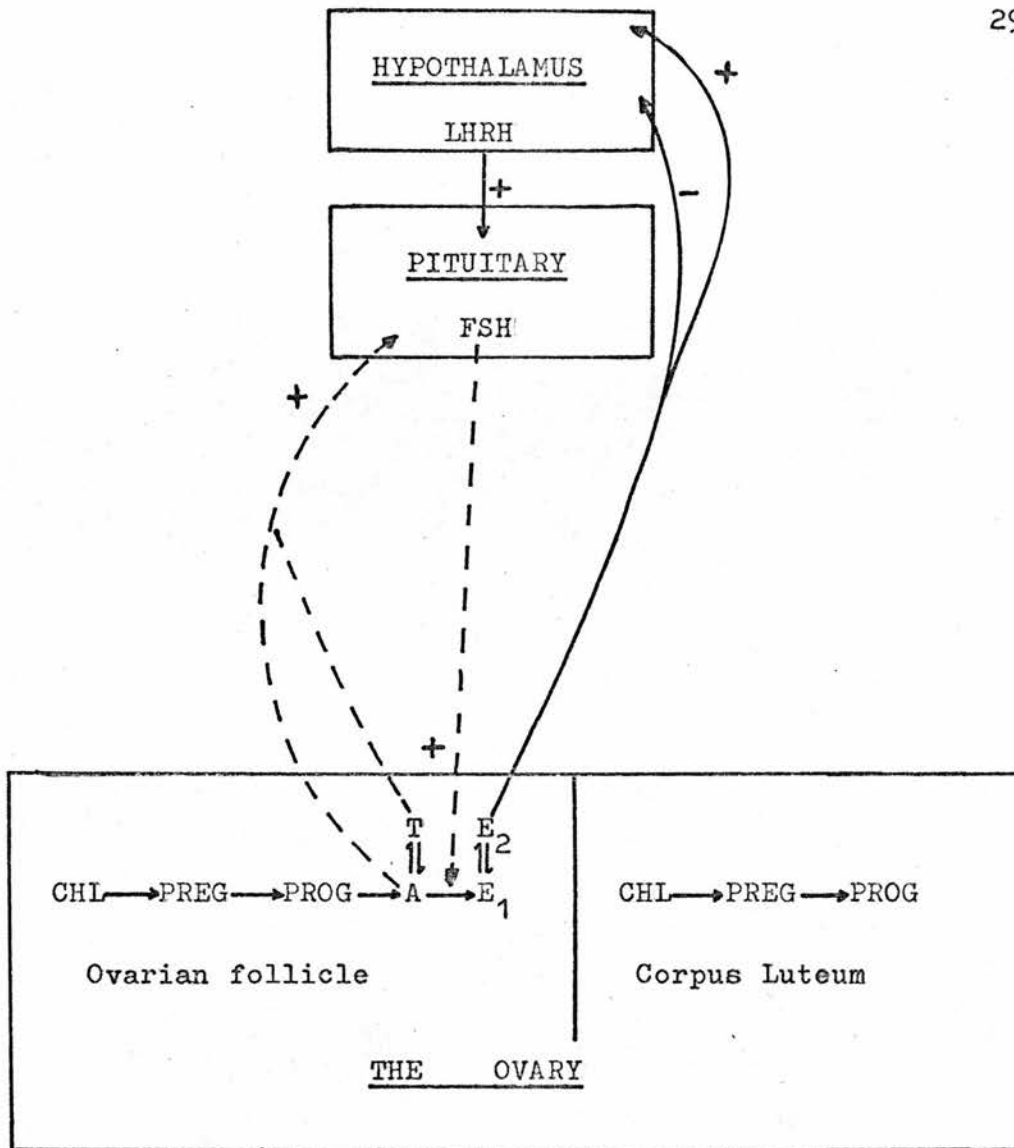


Figure 8.2.

THE CONTROL OF FSH SECRETION IN THE EWE

The unbroken lines represent accepted pathways of hormonal feedback control systems. The broken lines represent the postulated pathways of hormonal feedback action.

CHL = Cholesterol; PREG = Pregnenolone; PROG = Progesterone;

A = Androstenedione; T = Testosterone; E<sub>1</sub> = Oestrone;

E<sub>2</sub> = Oestradiol-17β; FSH = Follicle-stimulating hormone;

LHRH = Luteinizing hormone releasing hormone.

(+) = positive action. (-) Inhibitory action.

plasma concentration of FSH in the control ewes could be ascertained.

It seems likely, therefore, that although the secretion of LH and FSH may be governed primarily by the hypothalamic gonadotrophin-releasing hormone, other factors may serve to modify the release of gonadotrophins independently of one another, probably by a direct action at the pituitary level.

#### 8.3.2.1. The feedback actions of oestradiol on FSH secretion.

Oestradiol will inhibit the release of FSH in anoestrous ewes and also induce a positive discharge of FSH (fig. 8.2) (Jonas et al. 1973; Pant et al. 1973; Reeves et al. 1974a) in a situation analogous to the negative and positive feedback actions of this steroid on LH secretion. The present studies confirmed these earlier reports and demonstrated an initial depression of FSH followed by a discharge of FSH coincident with the corresponding alterations in the level of LH in anoestrus (fig. 4.2) and ovariectomized-hysterectomized ewes (fig. 4.8). It is interesting to note that a discharge of FSH in response to oestradiol was absent in an ovariectomized-hysterectomized animal even though a well-defined peak of LH was apparent (fig. 4.7), suggesting that the positive action of oestradiol on LH and FSH secretion could be mediated through independent mechanisms.

Active immunization against  $E_2$ -6-BSA produced a variable effect on the plasma concentration of FSH. In the two ewes with the highest oestradiol antibody titres (Table 5.1) the plasma concentration of FSH during anoestrus was significantly higher/

higher than the control value (fig. 5.7) and lay within the range of values found in the ovariectomized-hysterectomized animals (Table 5.3). It may be concluded that in these animals the circulating oestradiol antibodies had reduced the level of biologically active oestradiol sufficiently to lift the inhibitory action of this steroid on FSH secretion. A similar elevation in the plasma level of FSH (and LH) was found by Pant and Rawlings (1973) in ewes also actively immunized against  $E_2$ -6-BSA.

The three remaining oestradiol-immunized ewes, however, showed plasma levels of FSH which lay within the range of values found in the control animals (fig. 5.7). It may be argued that in the ewe, the release of FSH is more sensitive than the release of LH towards the inhibitory action of oestradiol, a situation thought to occur in humans (Tsai & Yen, 1971; Kulin & Reiter, 1972). In this case, the incomplete neutralization of oestradiol in these ewes may have resulted in the availability of a level of biologically active oestradiol sufficient to inhibit the more sensitive release of FSH.

#### 8.3.2.2. Do androgens influence the secretion of FSH in the ewe?

There is some evidence to suggest that androgens may exert a differential action on the release of FSH and LH. Low levels of testosterone may enhance the release of FSH from the rat pituitary in vitro without altering the release of LH (Mittler, 1972). A similar action for androgens was drawn from the finding that testosterone pretreatment enhanced the ovarian response to HCG/

HCG in immature rats (Johnson & Neqvi, 1969a,b; Neqvi & Johnson, 1969). Furthermore, it has been demonstrated that testosterone, in contrast to oestradiol, will enhance the synthesis of FSH in the castrated rat (Gay & Bogdanove, 1969) and may even antagonize the negative feedback action of oestradiol (Gay & Dever, 1971).

Perhaps the most surprising finding encountered during the course of the present studies was that whilst the level of LH was elevated in the androstenedione-immunized ewes, the plasma concentration of FSH was lower than the control value (figs. 4.6 and 4.9). This finding cannot be explained by an alteration in the secretion of oestradiol, a steroid known to suppress the release of FSH. The increased level of oestradiol necessitated by this explanation would be incompatible with the elevated level of LH.

At the risk of delving into the realms of pure hypothesis, two alternatives could contribute towards the presence of reduced levels of FSH in the androstenedione-immunized ewes. The first alternative may lie in the increased secretion of an ovarian "inhibin" recently postulated to exist in bovine follicular fluid (De Jong & Sharpe, 1976). This explanation awaits the confirmation of the existence of an ovarian inhibin in the ewe.

The second alternative may lie in a direct action of androgens on the release of FSH, probably by a direct action at the pituitary level. If androgens, at low levels, do enhance the release of FSH, thereby antagonizing the negative feedback action of oestradiol in the intact animal, then the absence of biologically active androstenedione could have resulted in an enhancement/

enhancement of the inhibitory action of oestradiol (fig. 8.2).

A similar explanation may underlie the presence of normal plasma levels of FSH in the ewes immunized against T-3-BSA. Although the presence of significant oestradiol antibody titres in these animals probably accounted for a pattern of LH release similar to that found in the two oestradiol-immunized animals with the highest oestradiol antibody titres, a corresponding elevation in the plasma FSH level was not observed (figs. 6.5 and 6.9). The testosterone-immunized ewes differed from the oestradiol-immunized animals by the presence of high titres of testosterone and androstenedione antibodies. The absence of biologically active androgens in the testosterone-immunized ewes may have enhanced the negative feedback action of any residual biologically active oestradiol.

It is unfortunate that the limited supply of the anti-ovine FSH serum used in the radioimmunoassay did not permit a more thorough examination of FSH secretion in the androgen-immunized ewes. Nevertheless, it is interesting to speculate on the physiological significance of the proposed action of androgens on the release of FSH. It has recently been demonstrated that FSH will increase the aromatization of androgen precursors to oestradiol within the rat ovary (Moon et al. 1975; Armstrong & Papkoff, 1976; Dorrington et al. 1976). If this hypothesis proves correct for events occurring in the ewe, then the increased ovarian secretion of androgens in response to LH (McCracken et al. 1969) through its action on the conversion of cholesterol to pregnenolone (Savard et al. 1965; Flint & Armstrong, 1972) would enhance the release of FSH. The increase in FSH could then serve to stimulate the aromatization of androgens/

androgens to oestrogens which in turn would suppress the secretion of LH and FSH.

It cannot be over emphasized that the proposed involvement of androgens in the release of FSH arose largely from the presence of reduced plasma concentrations of FSH in the androstenedione-immunized ewes and awaits further confirmation. The proposed system, however, offers a mechanism whereby the ratio of androgens to oestrogens secreted by the ovary may be controlled, a function which LH cannot perform through its action upon the initial stages of steroidogenesis.

#### 8.4. CONCLUSIONS.

The present investigation examined the effects of active immunization against bovine serum albumin conjugates of four steroids known to be secreted by the sheep ovary on gonadotrophin secretion in the non-pregnant ewe. Whilst these studies are limited by problems involved in the evaluation of both the specificity of the immune response and the degree to which the biological actions of the endogenous steroids are neutralized in vivo, it is possible to gain an insight into the mechanisms controlling gonadotrophin secretion in the ewe.

(1) Active immunization against  $E_2$ -6-BSA produced an elevation in the plasma concentration of LH during anoestrus and during the breeding season. A reduction in the level of biologically active oestradiol produced a pattern of LH release during anoestrus similar to that found in ovariectomized-hysterectomized ewes. The absence of behavioural oestrus during the breeding season further confirmed the reduction in biologically/

biologically active oestradiol. The blockade of ovulation in the oestradiol-immunized ewes was confirmed at laparotomy and the presence of multiple follicular development reflected the elevated levels of LH. The present study confirmed the dual actions of oestradiol on LH secretion as suggested by the actions of exogenous oestradiol in ovariectomized ewes.

(2) The close correlation between the ovarian secretion of androstenedione and oestradiol posed the question as to a possible role of androstenedione in the control of LH secretion in the ewe. The increased frequency of spontaneous discharges of LH together with the elevated basal level of LH during the luteal phase of the oestrous cycle in the androstenedione-immunized ewes suggested that the negative feedback control of LH secretion had been reduced. The absence of elevated plasma binding of oestradiol and the presence of normal oestrous behaviour indicated that the biological actions of oestradiol were unaltered by immunization against A-11-BSA.

A modification in the positive feedback action of oestradiol in the androstenedione-immunized ewes was suggested by the delay or absence of the LH surge following oestradiol treatment and also by the delay in the time of the preovulatory LH surge following the onset of oestrous behaviour during the breeding season. Corroborative evidence for this hypothesis arose from the finding that androstenedione implants enabled the induction of a surge of LH in response to a subthreshold dose of oestradiol during anoestrus.

It is postulated that androstenedione could modify the hypothalamic sensitivity towards the positive and negative feedback actions of oestradiol on LH secretion in the ewe. The action/



action of androstenedione on LH secretion may be due to its extra-ovarian conversion to oestrone.

(3) The feedback mechanisms controlling FSH secretion in the ewe have not been clearly defined.

Exogenous oestradiol exerted a dual action on the secretion of FSH in anoestrous and ovariectomized-hysterectomized ewes in a manner analogous to the negative and positive feedback actions of this steroid on the release of LH.

Immunization against  $E_2$ -6-BSA produced variable effects on the plasma concentration of FSH. In the two oestradiol-immunized ewes with the highest oestradiol antibody titres, the plasma level of FSH lay within the range of values found in ovariectomized-hysterectomized ewes. In the remaining three ewes, the level of FSH was not significantly different from the control value. If the secretion of FSH is more sensitive than the release of LH towards the inhibitory action of oestradiol then the incomplete neutralization of oestradiol may have left sufficient biologically active oestradiol to exert a significant inhibitory action on FSH secretion.

(4) There is some evidence, arising from studies in the rat, that androgens may enhance the release of FSH probably by a direct action at the pituitary level.

Active immunization against A-11-BSA produced a significant reduction in the plasma concentration of FSH. It is tentatively proposed that the reduction in the level of biologically active androstenedione may have enhanced the inhibitory action of oestradiol. Similarly, the neutralization of biologically active testosterone and androstenedione in the ewes immunized against T-3-BSA may have prevented an elevation in the level of FSH/

FSH which might be expected from the reduction in the level of biologically active oestradiol.

(5) The physiological significance of the proposed action of androgens on the release of FSH was discussed in the light of recent studies which suggest that FSH may enhance the aromatization of androgen precursors to oestrogens within the ovary. The enhanced ovarian androgen secretion in response to LH could enhance the release of FSH. The elevation in FSH would then enhance the aromatization of androgens thereby increasing the secretion of oestradiol which in turn would result in the suppression of LH and FSH secretion. The postulated control system could provide a physiological mechanism whereby the ratio of androgens to oestrogens secreted by the ovary may be regulated.

(6) The proposed action of androgens on the release of FSH was formulated largely from the presence of reduced plasma levels of FSH in the androstenedione-immunized ewes. Bearing in mind the limitations in the interpretation of immunological studies, the proposed feedback systems controlling gonadotrophin secretion in the non-pregnant ewe await further confirmation.

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